NONPRECIPITATING INSULIN ANTIBODIES[1, 2]

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Most immunologic tests currently in use depend upon the detection and quantitation of some result of the union of antigen and antibody. Such consequences of union include precipitation, agglutination, complement fixation and hemolysis. It is evident that the union of antigen and antibody precedes and is a necessary condition for the occurrence of these and other secondary phenomena. There have always been excellent reasons to believe that antigen could combine with antibody without the occurrence of such secondary reactions. A major difficulty in certain types of immunologic work in the past has been the necessity of determining the presence or the amount of antibody by measurement of the result of its union with the antigen rather than by the direct measurement of such a combination itself. A method which permits direct measurement of the union of antigen and antibody has obvious value for the study of systems where secondary reactions are either nonexistent or difficult to measure. The antibody which combines with insulin and which is the subject of this and the following paper is such an antibody. Its existence has long been suspected because of occasional local reactions to insulin and because of some of the biologic effects which the plasma of insulin treated patients has been known to possess. Until electrophoretic studies (1) demonstrated the combination of I^{131} insulin with gamma globulin of selected patients, there was no direct evidence of the existence of an antibody which combines with insulin in any human plasma. The recent work of Farr (2) demonstrating antibody with binding capacity for bovine serum albumin (BSA) stimulated the search for a similar method of demonstrating antibodies to insulin. A method of precipitating bound insulin with antihuman gamma globulin was finally selected. A similar method was used by Feinberg in a rabbit system (3).

MATERIALS AND METHODS

Antihuman gamma globulin (AHGG) was prepared in rabbits by repeated injections of human globulin either as a solution in 0.85 per cent NaCl or in a water-in-oil emulsion. The human gamma globulin (HGG) was obtained from The Cutter Laboratories and labeled with I^{131} (4). The titer of AHGG was determined either by estimating the equivalence point of the precipitin curve or more accurately by the labeled antigen precipitation method (5).

To 0.5 ml. of a 1:25 dilution of human serum was added 0.5 ml. of 1:40,000 dilution of I^{131} insulin containing 0.1 μg. insulin per ml. (from Abbott Laboratories). The specific activity of the I^{131} insulin was 0.5 mc. per mg. The mixture of serum and insulin was allowed to incubate two or more hours and AHGG was added. After standing in the cold overnight, the mixture was centrifuged for 30 minutes at 2,000 rpm, decanted, washed once, and the radioactivity of the precipitate and supernatant counted. All dilutions and washings were performed with a nine parts normal saline, one part borate buffer (pH 8.4) solution. All procedures were done in duplicate at 4° C.

Human anti-insulin serum was obtained from a patient receiving injections of insulin. This serum and I^{131} insulin were mixed in concentrations known to bind approximately 80 per cent of the insulin. Identical mixtures were prepared in two tubes. After incubation for two hours, sufficient rabbit AHGG was added to one tube to precipitate all the HGG. To the other tube was added an equal volume of normal rabbit serum. The mixtures were then centrifuged for one hour at 600 × G, and the supernatants fractionated electrophoretically on a starch block (6).

RESULTS AND DISCUSSION

If AHGG is added to HGG in a molecular ratio of 3:1, there is approximately 100 per cent precipitation of HGG (Figure 1, a). Insulin tagged with I^{131} was found to be soluble in AHGG, in any HGG, and in the supernatant of an AHGG and HGG mixture (Figures 1, b and c). When

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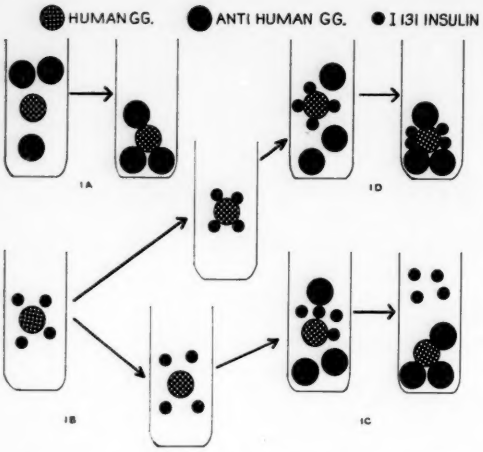

FIG. 1. DIAGRAMMATIC REPRESENTATION OF THE PRINCIPLE BY WHICH ANTIHUMAN GAMMA GLOBULIN (AHGG) DEMONSTRATES THE PREEXISTING UNION BETWEEN I^{131} INSULIN AND HUMAN GAMMA GLOBULIN (HGG)

HGG is precipitated by AHGG, insulin bound by HGG is found in the precipitate (Figure 1, d).

An experiment was performed to determine the relationship between the precipitation of gamma globulin and the demonstration of the insulin gamma globulin complex. Varying amounts of AHGG were added to constant mixtures of : 1) I^{131} insulin and a human anti-insulin serum from a patient treated with insulin; 2) trace amounts of I^{131} HGG and the same anti-insulin serum; and 3) I^{131} insulin and a normal human serum. The results in Figure 2 show that the identification of the gamma globulin-insulin complex depends upon the quantitative precipitation of the gamma globulin. The precipitation of globulin does not increase the binding of insulin; it merely demonstrates the already existing union.

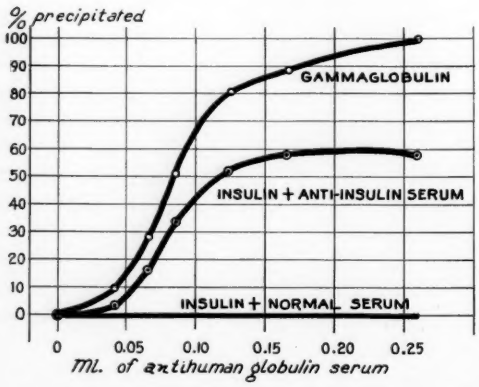

FIG. 2. DEMONSTRATION THAT MEASUREMENT AND IDENTIFICATION OF INSULIN-GAMMA GLOBULIN COMPLEXES DEPEND UPON THE QUANTITATIVE PRECIPITATION OF GAMMA GLOBULINS

The curve labeled gamma globulin refers to the per cent of labeled gamma globulin precipitated with each concentration of the antihuman gamma globulin added. The other two curves refer to the per cent of insulin demonstrated in the precipitate.

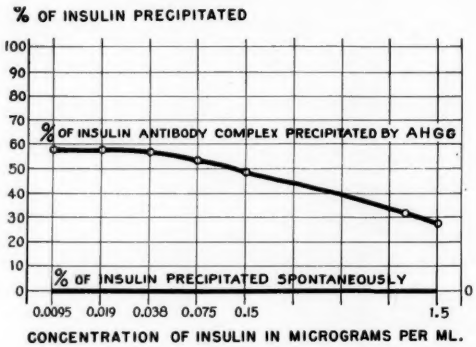

FIG. 3. EFFECT OF INSULIN EXCESS ON PER CENT BINDING

The percentage of insulin which is bound to gamma globulin is decreased although the total amount bound is increased.

In another experiment, varying amounts of I^{131} insulin were mixed with a constant amount of an anti-insulin serum before the addition of AHGG. The amount of AHGG added was that amount which was previously determined to precipitate 100 per cent of the gamma globulin in this serum. The time of incubation before addition of AHGG was two hours; longer incubation did not yield higher percentages of insulin bound. The results of this experiment as shown in Figure 3 indicate that the reaction between insulin and globulin behaves according to the mass action law. A fivefold increase in the amount of insulin added reduced the fraction of insulin precipitated by a factor of only two. The total amount of insulin bound increased, therefore, by a factor of two and one-half. Since the total quantity of insulin bound

was increased by increasing the insulin concentration, it is evident that there was incomplete saturation of binding sites at the lower concentration. Such an incomplete saturation of binding sites could occur at equilibrium in the presence of excess insulin only if the bond between insulin and globulin were reversible. This conclusion is in agreement with that of Berson, Yalow, Bauman, Rothschild, and Newerly (1), who demonstrated the reversibility of the insulin-globulin bond directly by an exchange between labeled and unlabeled insulin.

Figures 4 and 5 give the results of an electrophoretic separation of an insulin anti-insulin mixture before and after precipitation of the HGG with AHGG. The fact that precipitation of the I^{131} insulin-HGG complex by AHGG did not result in an increase in the amount of free insulin indicates that precipitation of the complex does not result in dissociation of the insulin from the globulin.

The precipitation of an antigen-antibody complex with an antiglobulin serum is a relatively simple method of demonstrating a nonprecipitating antibody. The same principle has been used to study the binding by selected human sera of I^{131} thyroxine (7) and C^{14} labeled progesterone (8). The absence of detectable binding in the latter case confirms the electrophoretic studies of Westphal (9). In addition, AHGG has been used to separate gamma globulin from Bence Jones protein in tissue culture in order to study the differential incorporation of C^{14} lysine into these products (10).

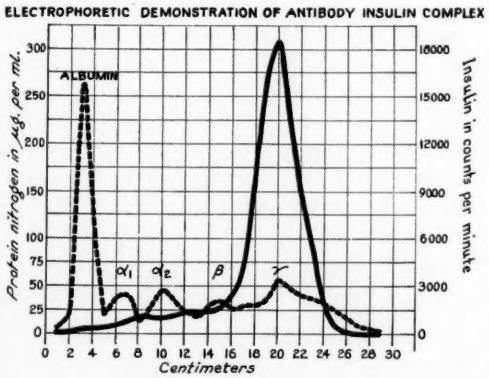

FIG. 4. ELECTROPHORETIC PATTERN OF I^{131} INSULIN ADDED TO SERUM CONTAINING INSULIN BINDING ANTIBODIES

The dotted line shows the electrophoretic migration pattern of this patient's serum protein. The solid line shows the migration pattern of I^{131} insulin with this serum on starch block. With a normal serum, unbound insulin migrates at approximately the same speed as albumin.

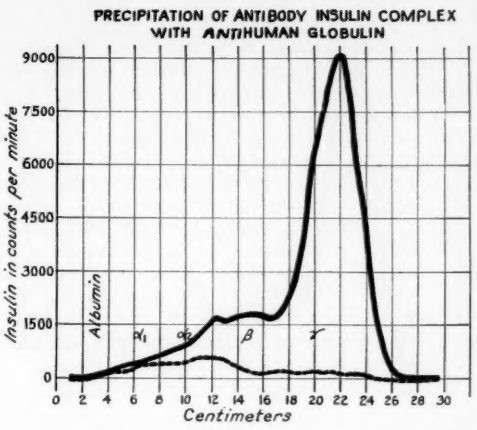

FIG. 5. ELECTROPHORETIC MIGRATION PATTERN BEFORE AND AFTER PRECIPITATION OF THE I^{131} INSULIN HUMAN GAMMA GLOBULIN COMPLEX

For the purpose of simplicity the serum protein curve is omitted. The solid line shows, as in Figure 4, the migration pattern of insulin bound to another patient's serum. The dotted line shows the electrophoretic distribution of the radioactivity remaining after the insulin-gamma globulin complex in this serum is precipitated by antihuman gamma globulin.

SUMMARY

A method is presented which measures the binding of insulin by human serum. The method is similar to that previously described by Feinberg in the use of an anti-gamma globulin serum to precipitate a soluble complex of antigen and gamma globulin. The method may be useful in demonstrating antibodies to other hormones.

ACKNOWLEDGMENTS

The authors wish to express their gratitude to Dr. Peter Stelos for performing the electrophoretic studies, and to Mr. William Cheng for his technical assistance.

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THE ROLE OF NONPRECIPITATING INSULIN ANTIBODIES IN DIABETES [1]

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(Submitted for publication November 13, 1957; accepted February 6, 1958)

Interest in antibodies to insulin developed with the genesis of the first reaction at the site of insulin injection (1–3). It was almost inevitable that once the existence of antibodies to insulin had been suggested, the effect of these on the insulin itself would be questioned. Studies performed on the serum of insulin resistant patients provided further impetus to the concept that antibodies to insulin inhibit the action of insulin, thus necessitating the use of of higher doses than would otherwise have been the case (1, 4–17). In some instances the methods then available could demonstrate antibodies to insulin by direct skin testing, by passive transfer, by complement fixation or by the use of collodion particles (2, 3, 18, 19). It was learned that skin sensitizing antibodies were present only in patients who had allergic reactions to insulin and were independent of the patient's insulin requirements (3–7, 18, 20). This appeared to indicate that if an antibody were present in the insulin resistant patient, this antibody was distinct from the skin sensitizing antibody. In most cases, this suspected antibody did not precipitate insulin, fix complement, hemolyze red cells or do anything else easily recognized. Therefore, the presence of this antibody had to be determined by neutralization or reduction of the physiological action of insulin.

The following experimental approaches have been used:

1. Treatment of insulin resistant patients with human insulin (6, 20).
2. Studies of the effect of the plasma of selected patients or animals on the uptake of glucose by a rat diaphragm, with and without added insulin (10, 15, 21–25).
3. Studies of the effect of selected plasma alone or with added insulin on animals which were also receiving an otherwise lethal or convulsive dose of insulin (5, 14, 15, 17, 20).
4. Studies of the effect of the plasma of selected patients on the degradation of insulin by rat slices and liver homogenates (21, 26).
5. Studies of the protective effect of gamma globulin from insulin resistant patients on animals receiving insulin (11–15).
6. Studies of the effect of the plasma of insulin resistant patients on an animal's blood sugar (8, 9, 13, 15, 16, 20, 27).
7. Studies of the effect of selected plasma and added insulin on the blood sugar of animals (8, 9, 11, 12, 16, 27).

As a result of these various experiments, it is apparent that the inhibition of insulin may occur through several different mechanisms, all of which are by no means clearly understood. These mechanisms are of three general types: a) cellular, *i.e.*, by competition with or antagonism of the cellular function of insulin; b) enzymatic destruction of insulin; and c) binding of insulin to circulating protein.

Since an enzyme is a protein which has a definite affinity for its substrate, insulinase might be considered an antibody if it occurred as a response to the injection of insulin. However, this response has never been demonstrated clearly and indeed insulinase activity has been found in sera of patients and animals that have never received insulin (27, 28). It is the third type of inhibition which is most clearly due to an antibody and is the subject of this study. The binding of insulin by serum has been demonstrated only in patients and animals that have received injections of heterogenous insulin. Indeed, it is a regular response to such injections (29, 30).

[1] This work was done with the support of Grant E-1378 from the National Institute of Allergy and Infectious Diseases of the National Institutes of Health.

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MATERIALS AND METHODS

In the preceding paper (31), a method was presented for demonstrating the binding of insulin by the gamma globulins in any given serum. Using this method the insulin binding by the serum of 48 patients was determined. The term "insulin binding" is used to designate the percentage of insulin which is bound by the gamma globulins of an individual serum under standardized conditions (31). Each serum was analyzed within 48 hours after it was obtained and then frozen at − 20° C. Since the concentrations of insulin in different lots of I^{131} insulin may vary somewhat, all of the results presented here were obtained from a simultaneous reanalysis of all the sera. The final and initial analyses did not differ in any case by more than 10 per cent. If less than 1 per cent of the radioactive insulin was bound by a 1:25 dilution of the individual serum, then this serum was classified in this paper as negative. Otherwise the actual per cent of insulin bound is given. Since the percentage binding is a result of both the amount of antibody present and the equilibrium constant of the antigen-antibody reaction, the results should not be interpreted as indication of the absolute amount of antibody present. However, due to the relatively slow turnover of globulins in humans, any sudden change in binding should reflect a similar change in the concentration of antibody. The amount of I^{131} insulin added under the conditions of the test is equivalent to 66 units per liter of undiluted serum. Since the amount of unlabeled insulin contained in the serum will, under almost any conceivable condition, be small in comparison to the labeled insulin added, and since the

TABLE I

Insulin binding of sera from insulin treated patients

Patient	Age	Sex	Insulin binding *per cent*	Insulin treatment *duration*	Daily dose of insulin in units			Comment
					Highest	Lowest	Most recent	
K. J.	43	F	0	16 days	20	5	5	No insulin for 2 mos. at time of study.
R. H.	65	F	0	3 days	15	10	10	
			0	14 days				
			0	18 days				
L. R.	64	F	0	6 yrs.; then none for 6 yrs.			15	Serum studied 9 days after insulin was resumed.
A. M.	67	F	2	6 yrs.+	125	30	45–55	Forty-five years of diabetes.
R. A.	66	M	2	9 yrs.	60	30	40–45	Steroids 5 mos. Insulin requirements increasing.
A. G.	27	F	2	6 yrs.	125	50	90–100	
A. G.	64	M	3	6 mos.	20	20	20	
H. B.	43	M	3	1 mo.; discontinued for 1½ yrs.	50	20	30–45	Serum studied 11 days after resumption. Insulin requirements increasing.
P. S.	46	M	6	5 mos.	30	0	15–20	Third sample 4 mos. later showed titers rising.
			4	5 wks.				
J. B.	55	M	8	7 yrs.	30	15	15–30	Three sera. Requirements decreasing. Fasting and nonfasting determinations show equal titers.
G. S.	35	F	12	8 yrs.	80	25	25–35	Titer going up although insulin requirements decreasing.
			18					
			22					
F. D.	56	F	8	4 yrs.; then none for 4 days; then 2 days' treatment	275	?	35–45	Insulin requirements decreasing. Fasting and nonfasting determinations show equal titers.
H. R.	32	M	16	14 yrs.	45	25	30–45	
O. J.	53	M	17	7 yrs.	15	10	10–15	Irregular dosage.
M. G.	24	M	17	17 yrs.	?	?	35–40	Juvenile diabetic. Dosage decreasing. Patient uremic at time of study.
F. R.	50	F	21	6 mos.	40	26	30–40	
J. A.	17	F	24	4 yrs.	91	25	25–40	Juvenile diabetic. Requirements decreasing.
H. M.	65	F	26	3 yrs.	?	?	13–15	Fasting and nonfasting titers are equal.
S. S.	66	M	28	3½ yrs.	120	12	90–120	Requirements increasing.
F. H.	45	F	36	13 yrs.	600	30	60–80	Lawrence-Ziegler syndrome. Requirements decreasing.
L. R.	52	M	39	12 yrs.	80	10	60–80	Decreasing requirements. Many insulin reactions.
H. K.	48	M	47	30 yrs.	?	?	50–65	Juvenile diabetic. Requirements decreasing.
P. G.	75	M	56	? 10 yrs.	50	30	40–50	Hemochromatosis and hepatoma.
E. P.	53	F	See Figure 1					
S. I.	58	F	See Figure 2					

TABLE II

Insulin binding of sera from insulin treated patients whose insulin had been discontinued

Patient	Age	Sex	Insulin binding *per cent*	Insulin treatment *duration*	Daily dose of insulin in units			Comment
					Highest	Lowest	Most recent	
W. T.	56	M	0	Less than 2 mos.	10	5	0	No insulin for 2 mos. at time of study.
W. S.	72	M	0	4 mos.	10	5	0	No insulin for 1 yr. at start of study. Four samples over 6 mos. period although patient developed fever, furuncles and hyperglycemia.
E. B.	60	M	0	2–3 yrs.	?	?	0	No insulin for 1½ yrs. at time of first study. Eight mos. later serum still negative.
L. M.	56	F	0	3 yrs.	?	?	0	No insulin for 10 mos. at time of study. Patient's blood sugar was out of control with infection and fever at this time.
J. K.	44	M	3	7 mos.	30	0	0	Lente insulin. None for 2 wks.
R. A.	50	F	3	4 yrs.	20	0	0	No insulin for 3 yrs. Three subsequent sera show the same results.
R. C.	57	F	6 (8/31) 4 (11/20) 3 (12/4) 6 (3/30)	7 yrs.	30	0	20–30 0 0 5–30	Determinations on 8/31, 11/20, 12/4 and 3/30. Decreasing insulin requirements with insulin stopped on 9/21 and resumed on 3/24.
R. K.	59	F	7	13 yrs.	20	0	0	Insulin stopped 7 wks. prior to first determination; repeated 2½ mos. later and still 2+.
I. T.	56	F	8	3 yrs.	40	0	0	No insulin for 3 years.
E. C.	60	F	18 10 9	3 yrs.	20	5		First serum studied after 23 days of insulin following 8 mos. of no insulin. Second serum was studied 2½ mos. after insulin was again discontinued. Third serum studied 3½ mos. later when patient still not on insulin.

labeled and unlabeled insulin exchange freely (29), it seems unlikely that the prior injection of unlabeled insulin will have an appreciable effect on the result of the test.

RESULTS

Table I shows the relative insulin binding by the sera of 25 diabetic patients currently receiving insulin. The insulin dosage, duration of treatment and other pertinent clinical information are presented in abbreviated form. No clear relationship is apparent between the dosage of insulin or duration of treatment and the insulin binding of the patient's serum. However, 22 out of the 25 sera were positive. Two of the negative sera had received insulin for 18 days or less. One had received insulin for only 5 days following 6 years without treatment. One patient's serum began to bind insulin as early as 5 weeks after the onset of treatment.

Table II presents similar data for patients whose insulin has been discontinued, with or without reinstitution of insulin. It may be seen that in some patients the antibodies persist as long as three years after treatment has been stopped. However, the average insulin binding of the sera in this group is lower than in the group currently receiving insulin (Table I).

In two patients (P. S. and G. S.), a rise in insulin binding with duration of insulin treatment was observed despite a reduction in the insulin dosage. No differences could be demonstrated at different times of the day although blood sugar levels (and presumably exogenous insulin concentration) vary.

No evidence was obtained of a nonspecific anamnestic rise in insulin binding as a response to fever or infection, as suggested by Steigerwald and Spielmann (32). In other respects, however, these findings are consistent with both the time required and the individual variation observed in many other antigen-antibody systems.

Figure 1 shows the nonfasting and some fasting blood sugars, the daily insulin dosage and the insulin binding capacity of the sera from a 57 year

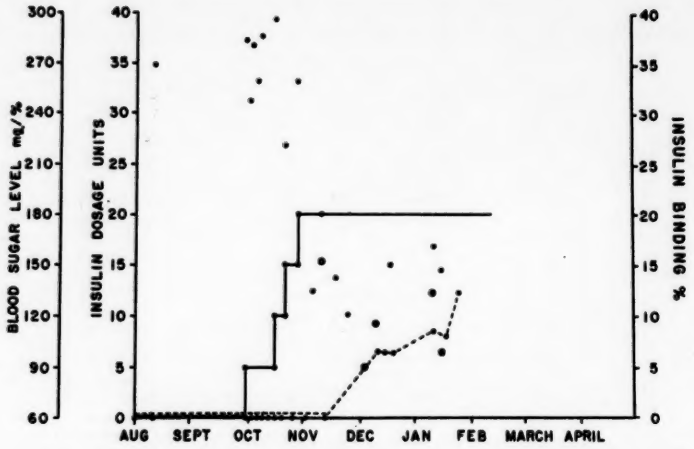

FIG. 1. INSULIN DOSAGE AND INSULIN BINDING OF THE SERA OF A DIABETIC PATIENT ON WHOM OBSERVATIONS WERE MADE FOR TWO MONTHS BEFORE BEGINNING OF THERAPY

The unbroken line represents insulin dosage, the broken line insulin binding, the isolated points nonfasting blood sugar, and the circles fasting blood sugar.

old Negro female who refused insulin for several months after diabetes was discovered. The time of development of insulin binding and its increase in titer in the face of reduced blood sugar are clearly demonstrated.

Figure 2 shows the fluctuations of the blood sugar of a 67 year old white female. The decrease in her insulin binding at a time when she was critically ill is demonstrated, as is the subsequent rise despite reduced insulin requirement and dosage.

Table III presents the negative controls studied. All are individuals who had never received insulin. These include one patient with hemochromatosis,

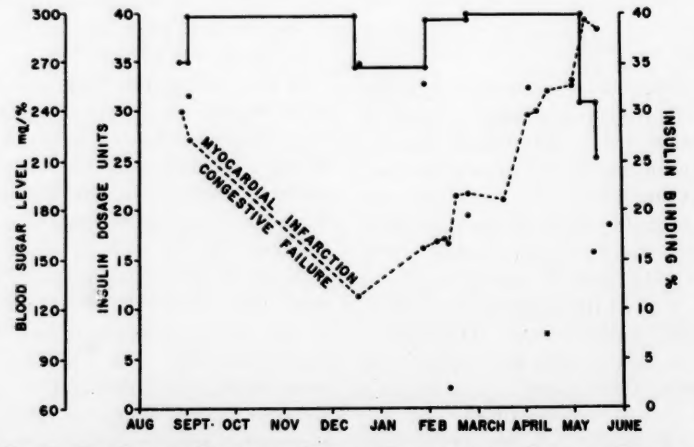
BLOOD SUGAR LEVEL mg/%
INSULIN DOSAGE UNITS
INSULIN BINDING %
MYOCARDIAL INFARCTION
CONGESTIVE FAILURE
AUG SEPT OCT NOV DEC JAN FEB MARCH APRIL MAY JUNE

FIG. 2. THE INSULIN DOSAGE AND INSULIN BINDING OF THE SERA OF A DIABETIC PATIENT WHO WAS OBSERVED DURING A PERIOD OF CRITICAL ILLNESS

The unbroken line represents insulin dosage, the broken line insulin binding, and the isolated points nonfasting blood sugar.

one with a functioning islet-cell carcinoma with metastasis, two healthy subjects and several patients with various diseases. Two of these patients had very low serum gamma globulin values and a few had elevated total globulins. None of them demonstrated any insulin binding.

DISCUSSION

The findings of the present work may be summarized as follows:

1. No insulin binding was found in patients, diabetic or otherwise, who had not received insulin.

2. Insulin binding was present in all of 23 patients who had received insulin recently for more than one month.

3. Antibodies were demonstrable in two patients who had received no insulin for three years.

4. An increase in insulin binding of a patient's serum was never found to precede or immediately follow an increase in insulin requirement or dosage.

5. No consistent relationship could be demonstrated between the insulin binding and the insulin requirements of patients receiving insulin.

On the basis of the evidence presented in this and the preceding paper, it would appear that the insulin binding demonstrated by precipitation with anti-human gamma globulin is due to the same antibodies which have been demonstrated electrophoretically (13, 29). The finding that the binding of insulin could be demonstrated only in the sera of patients treated with insulin strengthens the conclusion of Berson, Yalow, Bauman, Rothschild, and Newerly (29) that the globulin responsible for this binding should be considered an antibody. It does not necessarily follow, however, that the affinity of insulin for the antibody is unchanged by the I^{131} label.

The lack of relationship between insulin binding and the insulin requirements of the patient is in apparent contradiction to the recent reports of Berson and Yalow (30, 33) and Burrows, Peters, and Lowell (34) that the sera of insulin resistant patients show much greater binding of insulin than do the sera of nonresistant patients. These authors found that the sera of insulin resistant patients bound 80 to 800 units of insulin per liter of serum, whereas nonresistant patients' sera rarely bound more than 10 units. Under the conditions of the present study (31), 100 per cent binding of the insulin would represent 66 units bound per liter of undiluted serum. Since the test was performed with a 1:25 dilution of serum, the insulin bound in undiluted serum would be higher than this because of the mass action effect. Under the conditions of the test, the serum of patients who had received insulin for more than five weeks bound from 2 to 56 per cent of the insulin, which represents 1 to 37 units of insulin bound per liter of serum. It is evident from this data that considerable binding of insulin may occur without marked insulin resistance. Unfortunately, no patient with an insulin requirement above 100 units per day was included in this study, since the data of Burrows and co-workers and Berson and associates suggest that unusually high binding capacities may contribute to insulin resistance. An alternative explanation of their data, given by Lazarow in the discussion following Reference 30, is that the high levels of antibody are the response to the large and repeated injections of insulin. Repeated determinations of insulin binding on the same patient through numerous changes in insulin requirement may help resolve this problem of which factor is cause and which is effect.

A partial explanation of the relative lack of effect of insulin antibody on the insulin requirements of the diabetic patient has been given by Yalow and Berson (35) in the inhibition of liver insulinase activity by serum containing insulin binding antibody. In contrast to the effect of antibody in accelerating antigen breakdown in some systems (36), the insulin antibody appears to retard antigen metabolism (29). Just as the

TABLE III

Twelve patients who have never received insulin, all without demonstrable insulin binding

Patient	Age	Sex	Comment
F. S.	60	M	Hemochromatosis, diabetes.
F. T.	61	M	Arteriosclerosis, diabetes.
S. W.	61	F	Functional islet cell adenocarcinoma with metastases.
B. W.	45	M	Blood dyscrasia and acquired hypogammaglobulinemia.
T. H.	50	M	Hypogammaglobulinemia.
F. H.	52	M	Normal male.
A. A.	55	M	Asthmatic bronchitis.
P. E.	62	M	Cerebral arteriosclerosis.
H. L.	56	M	Gouty nephritis, hypertension, congestive failure.
W. C.	21	M	Normal male.
M. P.	30	F	Allergic asthma.
M. S.	52	F	Myxedema.

binding of insulin in a protamine precipitate does not increase total insulin requirement, the binding by antibody will not increase total insulin requirement if 1) the breakdown of the insulin is not accelerated, and 2) if either of the following obtains: *a*) the insulin-antibody bond is reversible, or *b*) the insulin-antibody complex is physiologically active. No information is available concerning the activity of the insulin-antibody complex, but evidence for the reversibility of the insulin-antibody bond has been given by Berson and Yalow (*29*) and by Skom and Talmage (31). Therefore, the two most apparent explanations for the relative lack of effect of antibodies on the insulin requirements of the patient are: 1) the failure of insulin antibodies to accelerate the metabolism of insulin, and 2) the reversibility of the insulin-antibody bond. As the free insulin is utilized by the cells and the free insulin concentration falls, the complex dissociates more rapidly than it forms, yielding more free insulin. As long as the binding of insulin is only temporary and does not lead to its accelerated metabolism, no increase in insulin requirements will result.

In contrast to the relative ineffectiveness of binding antibody in increasing the long term insulin requirement of the patient, binding antibody would be expected to increase the dose of insulin required for a given immediate effect. An example of this may be the increasing dosage of insulin required to induce shock in a schizophrenic patient receiving repeated injections. Berson and Yalow (30) suggest that the total effect of the insulin is not reduced by the presence of antibody in such cases but only delayed so that the hypoglycemia may be more prolonged.

SUMMARY

1. The insulin binding of the sera of 48 subjects has been determined.

2. Insulin binding was demonstrated in the sera of all 23 patients who had received insulin recently for more than one month.

3. Twelve control sera from patients who had never received insulin were all negative.

4. No consistent relationship could be demonstrated between the insulin binding and the insulin requirements of patients receiving insulin. The explanation of this relative lack of effect of insulin binding antibodies has been discussed.

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ADRENOCORTICAL HYPERFUNCTION IN "IDIOPATHIC" HIRSUTISM AND THE STEIN–LEVENTHAL SYNDROME [1]

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This is a report on a series of 13 women with hirsutism who have been studied with regard to the isolation, characterization and quantitation of their major urinary ketosteroid metabolites. No attempt was made to select the subjects on any basis other than obvious excessive hair growth. By the methods employed (1, 2), it has been possible to demonstrate the existence of a specific quantitative alteration in the steroid production of these hirsute women which, as a group, distinguishes them from normal women. The direction and character of this alteration permits the conclusion that in these subjects there was an excessive elaboration of adrenal steroid hormones. Correlation of the presence of hirsutism with known pathways of steroid metabolism in man leads to the interpretation that this excessive hormonal production was in larger measure the adrenal androgen.[2] A relationship may then exist between a particular type of cortical hyperfunction and hypertrichosis in certain of these women. This conclusion confirms and extends that reached previously in a detailed study of a single patient (3).

Four women with hirsutism, in whom it was later possible to establish the diagnosis of the Stein-Leventhal syndrome, are included in this study. The results of these women were taken into account because the sole criterion of patient selection was hirsutism and there was no doubt about this feature. Since the diagnosis of Stein-Leventhal syndrome was unequivocally established only by direct examination of the ovaries, these patients would have been classified as exhibiting hirsutism of undetermined cause if they had not been subjected to laparotomy. Since adrenocortical hyperfunction was evident in each, the results may be considered to support previous speculation concerning the role of the adrenal cortex in the expression of certain of the clinical manifestations of this disorder (4–6).

SUBJECTS AND METHODS

Thirteen consecutive women seen in the Endocrine Clinic of Memorial Hospital with the problem of hirsutism were studied. They are identified by initial and age. The following urine collections were studied: P17, a 10 day control; L21, a 5 day control and a 1 day period representing the second day of administration of cortisone, 75 mg. per day; G24, a 3 day control and a 5 day period on cortisone administration, 50 mg. per day; S24, a 7 day control and a 1 day period representing the second day on hydrocortisone, 120 mg. per day; C25, a 5 day control; B25, a 5 day control; K28, a 5 day control; A28, a 2 day control; R32, a 10 day control; C32, a 12 day control; M45, a 1 day control; M50, a 5 day control and a 5 day period representing the last half of a 10 day period on cortisone, 100 mg. per day; M62, a 9 day control and a 4 day period representing Days 6 through 9 on cortisone, 75 mg. per day. Two normal young women [Subjects M22 and W25 from a previous investigation (2)] received 50 mg. of cortisone daily for 6 days.

All patients were referred for study because of long-standing generalized hirsutism of undetermined cause. Breast development was variable but there were no pronounced alterations of body habitus in any subject. There was moderate clitoral enlargement in several but external genitalia were otherwise normal. Sterility was not a problem in the several married patients studied. Significant disturbances of the menstrual pattern occurred in only four subjects, P17, L21, S24 and C25. These women complained of severe oligomenorrhea or prolonged periods of amenorrhea. Clinical study here and elsewhere failed to disclose a discrete lesion as a basis for hirsutism in any patient. Because of suggestive clinical features including elevated steroids this

[1] This investigation was supported in part by a grant from the American Cancer Society and research grants (CY-3207 and C-925) from the National Cancer Institute of the National Institutes of Health, United States Public Health Service.

[2] The term "adrenal androgen" is a convenient designation for the steroid hormone (or hormones) produced by the adrenal and metabolized predominantly to androsterone and etiocholanolone. The chemical nature of this component of the adrenal secretion is under active investigation in these laboratories.

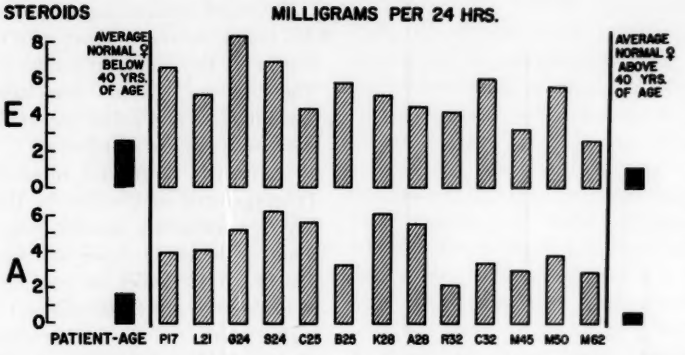

FIG. 1. PRODUCTION OF ANDROSTERONE (A) AND ETIOCHOLANOLONE (E) BY HIRSUTE WOMEN COMPARED WITH AVERAGE VALUES OF NORMAL WOMEN

study included laparotomy in P17, L21, G24, C25, A28, M45 and M50, in order to eliminate the possibility of adrenal or ovarian tumors. An autopsy examination was performed on M62, who died some time after study. Adrenals and ovaries were felt to be grossly or microscopically normal in all subjects except P17, L21, S24 and C25. In these four women, bilaterally enlarged polycystic ovaries were found similar to those described by Stein and Leventhal (7).

Urine collections were complete as judged by the daily creatinine excretion. As soon as practicable after collection the urinary conjugates were hydrolyzed with enzyme and acid according to Method E (2). Preparation of α and β ketonic subfractions was done as previously described (1). The separation of individual components was accomplished exclusively by paper chromatography (2), with identification by means of infrared spectrometry where necessary. The values for each compound are corrected from equivalents of dehydroisoandrosterone to mg. of the substance measured. Results for each compound are shown in mg. per 24 hours. The average values for normal women are calculated from data published previously (2), which have been confirmed by current studies both as to the range of values reported as well as the depression in 11-desoxy-17-ketosteroids with age. The following abbreviations are used: A for 3α-hydroxyandrostane-17-one (androsterone); E for 3α-hydroxyetiocholane-17-one (etiocholanolone); OH-A for 3α,11β-dihydroxyandrostane-17-one; OH-E for 3α,11β-dihydroxyetiocholane-17-one; 11 = OE for 3α-hydroxyetiocholane-11,17-dione; D for 3β-hydroxy-Δ^5-androstene-17-one (dehydroisoandrosterone).

RESULTS

Figures 1 and 2 show the values for each of the isolated steroids from the hirsute women and average values for normal women below and above 40 years of age. Production of A and E by hirsute women in every case exceeded that of normal women of comparable age; in most instances the amounts were comparable to those found in normal men (Figure 3). Considered as a group, the production of A plus E in the hirsute women was more than three times that of the average of the normal women of all ages. The average production of A plus E by the hirsute group below 40 years of age was two and one-half times that of the

PATIENT-AGE		P17	L21	G24	S24	C25	B25	K28	A28	R32	C32	M45	M50	M62	
STEROIDS	WOMEN UNDER 40 YRS.	AMOUNT IN MILLIGRAMS													WOMEN OVER 40 YRS.
11=OE	0.5	1.5	0.7	0.8	0.9	0.8	1.4	1.3	0.7	0.6	0.2	0.4	0.6	0.8	0.5
OH-E	0.4	0.7	0.8	1.0	0.8	0.3	1.0	1.1	0.8	1.0	Tr.	0.2	0.7	0.9	0.6
OH-A	0.5	1.3	1.4	0.7	0.7	0.8	1.4	1.6	1.0	0.8	Tr.	0.3	0.6	1.4	0.5
D	0.3	5.9	0.6	0.2	1.4	2.1	4.6	0.9	2.4	0.1	5.7	0.4	0.5	0.2	0.2

FIG. 2. PRODUCTION OF MAJOR STEROID HORMONE METABOLITES BY HIRSUTE WOMEN COMPARED WITH AVERAGE VALUES FOR NORMAL WOMEN BELOW AND ABOVE 40 YEARS OF AGE

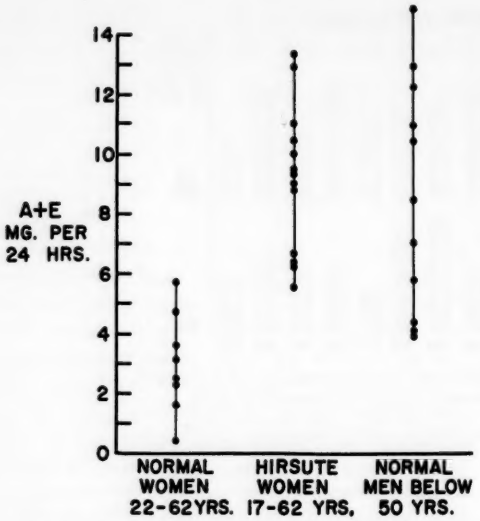

FIG. 3. RANGE OF VALUES FOR ANDROSTERONE PLUS ETIOCHOLANOLONE (A PLUS E) FOR HIRSUTE WOMEN COMPARED WITH NORMAL MEN AND WOMEN

average for the normal women in this age category. The increases in these compounds were variable, ranging from the minimal alterations shown by R32 to the pronounced elevations found with Subjects G24, S24, and K28.

The values for dehydroisoandrosterone (D) are in Figure 2. There is a greater variability in the excretion of this compound by both normal and hirsute subjects than there is with any of the 11-oxygenated adrenal metabolites.

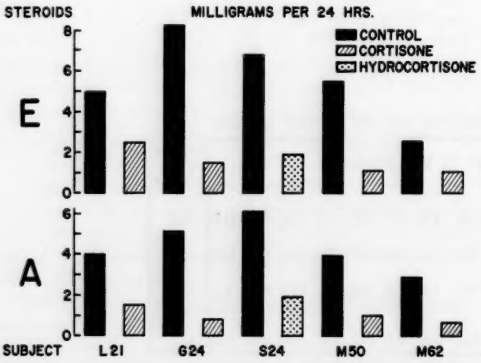

FIG. 4. EFFECT OF CORTISONE AND HYDROCORTISONE ON THE PRODUCTION OF ANDROSTERONE (A) AND ETIOCHOLANOLONE (E) BY HIRSUTE WOMEN

Extended methodological surveys indicate that this is not the result of error in technique but the reason for the scatter in results is not understood. There is no obvious correlation between the amount of D and either the 11-desoxysteroids or the 11-oxygenated metabolites.

There was, in general, increased production of 11-oxygenated metabolites by the hirsute women but of a definitely smaller magnitude than that found with the 11-desoxysteroids. Thus, OH-E and 11 = OE (which are considered to be derived principally from hydrocortisone) were 50 per cent higher than the level characteristic of normal women; OH-A (which is only a minor product of hydrocortisone metabolism) was 80 per cent higher than the normal level.

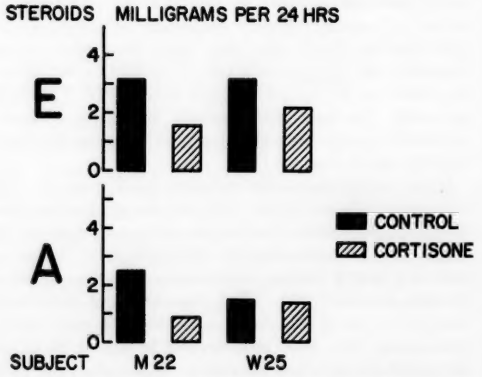

FIG. 5. EFFECT OF CORTISONE ON THE PRODUCTION OF ANDROSTERONE (A) AND ETIOCHOLANOLONE (E) BY NORMAL WOMEN

The effect of exogenous cortical steroid on the production of 11-desoxyketosteroids of the hirsute women is shown in Figure 4. There was a significant decrease in the production of these compounds by all subjects. The effect of cortisone administration on A and E production in two normal young women is shown in Figure 5. The results charted are for the third day of cortisone feeding; there was no material alteration in these values by the sixth day.

DISCUSSION

Examination of the individual urinary steroids revealed quantitatively significant alterations from a normal pattern in each of the hirsute women

studied. This excessive steroid production is of considerable interest since all of the subjects presented hirsutism but were an otherwise quite heterogeneous group. The detailed results were to a certain extent foreshadowed by scattered reports that "ketosteroids" are sometimes elevated in simple hirsutism (8). Separation of the individual components of "ketosteroids" as in this study, however, permits a specific and plausible interpretation of the endocrine aspects of this disorder. It is emphasized that hirsutism was the only criterion of patient selection. The inclusion of four patients with a later established diagnosis of the Stein-Leventhal syndrome was, in this respect, fortuitous.

The principal steroid alteration demonstrated was an elevated production of androsterone and etiocholanolone (A and E). Each of the hirsute women produced abnormally large amounts of these two substances when compared with women of commensurate age; indeed, the amounts found equaled the production of these steroids by young normal men. The significance of this fact is apparent when it is considered that in addition to a probable sex difference in adrenal function, the male gonad definitely contributes a portion of these metabolites.

These observations on steroid production by hirsute women lead necessarily to a consideration of the source, nature and biological properties of the endogenous precursors of these steroids. The evidence is substantial that in women as well as in men the adrenal cortex elaborates the principal precursors of the urinary metabolites studied (2, 9). Among the various facts that can be cited for the adrenal origin of the hormones which are metabolized to A and E the following are pertinent: 1) Castrated men and women with intact adrenals continue to produce both A and E (9). These two metabolites, however, are not found in the urine of adrenalectomized and castrated human patients. 2) Women and men markedly increase the production of A and E when stimulated with adrenocorticotrophic hormone (ACTH) (2, 10). 3) Normal men and women who have received suppressive doses of cortisone exhibit a significant diminution in these metabolites (10). All these are explicable only in terms of an adrenocortical origin of the hormones that give rise to A and E. With specific reference to the origin of A and E in the hirsute women examined in this study, the adrenal cortex is demonstrably involved since a fall in production of these compounds to levels observed in normal nonhirsute women was readily achieved with cortisone or hydrocortisone (Figures 4 and 5). The suppression of adrenal activity by cortisone or hydrocortisone administration showed that the hypersecretion of androgen in these women was under pituitary control but whether the adrenal was hypersensitive or the ACTH production was excessive is neither evident nor is it relevant to the conclusions drawn from this study. It should be noted, however, that depression of adrenal function by exogenous steroid occurred in the Stein-Leventhal syndrome (L21, S24) contrary to previous report (11).

From the considerations outlined it may be assumed that the elevated level of A and E in the hirsute women reflected the existence of adrenocortical hyperfunction. The nature of this excessive adrenal secretion can be described in the following terms. The steroid metabolites isolated in abnormal amount were the same compounds that result from the metabolic transformation of known androgens such as Δ^4-androstene-3,17-dione, dehydroisoandrosterone or testosterone (12–14). Similarly, the principal clinical feature in these women was unmistakably androgenic. Thus both the clinical evidence and the chemical structure of the metabolites are compatible with the interpretation that the adrenocortical secretion in these subjects was characterized by an excessive production of adrenal androgen.

There were other chemical manifestations of adrenal hyperactivity, evidenced by an elevated production of C_{19}-11-oxygenated steroids in several women. In this latter group, together with the increased hydrocortisone metabolites 11 = OE and OH-E, most exhibited abnormal amounts of OH-A, a steroid metabolite of 11β-hydroxy-Δ^4-androstene-3,17-dione (15) which is present in human adrenal vein blood (16). This latter substance is itself weakly androgenic. Thus, the increased adrenal function was not limited to metabolites of 11-desoxysteroid hormones even though this class showed the greater elevation.

The demonstration of adrenocortical hyperactivity in four women with the Stein-Leventhal syndrome is of considerable interest. Previous attempts to study alterations in the adrenal secre-

tion in this disorder (17) must be reviewed in the light of advances in steroid analysis which permit the recognition of less overt derangements in glandular physiology than are possible by the ordinary clinical assay of neutral "17-ketosteroids." It seems likely that more detailed steroid analyses of the type employed in the present study would reveal significant hormonal alterations in a number of these women. Indeed, these biochemical observations, together with the association of polycystic ovaries with frank adrenal disease (4, 18) and the frequent occurrence of hirsutism in the Stein-Leventhal syndrome (19), suggest that altered adrenal function, as described here, might be a significant or even primary influence in this disorder.

Accumulated evidence has led to the interpretation that the secretion of the adrenal cortex in the human contains at least two major groups of steroid hormones, either of which can be suppressed or stimulated without material effect upon the other. The experimental basis for this conclusion has been summarized (20, 21). In terms of this hypothesis, the biochemical lesion defined by these studies of hirsute women is an adrenal hyperfunction largely limited to the adrenal androgen; hyperactivity of the biosynthetic mechanisms leading to hydrocortisone production, on the other hand, is only a minor feature of this syndrome. If it be granted that increased production of A and E has been demonstrated in this study, it may appear mildly surprising that this is not accompanied by an increased secretion of hydrocortisone and chemically related hormones. Quite apart from the hypothesis presented above, however, the physiological consideration must be borne in mind that *these hirsute women do not exhibit signs and symptoms of Cushing's syndrome.* If the adrenal hyperfunction were other than the unilateral type proposed, it would necessarily follow that some evidence of hydrocortisone excess would be manifest clinically. That this is not generally the case may be considered confirmatory evidence for the explanation advanced.

The biochemical-clinical correlation described in these patients with hirsutism is suggestive of a causal relationship between the abnormal adrenal secretion and the hypertrichosis. Indeed, if the metabolic fate of the adrenal androgen were similar to that of testosterone in man (12, 22), the excessive secretion in these subjects would bring their hormone production into the range of normal males. Such hormonal influence operating constantly over a prolonged period of time seems entirely capable of stimulating and supporting the hypertrichosis in these patients. It is apparent that the unknown biologic potency of the adrenal androgen, and the variety of factors, including genetic ones, that might influence tissue response to hormonal stimulation, are considerations of importance in the interpretation of these data. Moreover, despite the uniformity of the observations in this study it is not certain that the hormonal aberration demonstrated will invariably be evident in all women with idiopathic hirsutism since extrahormonal influences must be variable and complex. Nevertheless, the observations described in this report are definitive of significant adrenocortical hyperfunction and indicate the existence of an important influence on the growth of hair in a considerable number of these patients.[3]

SUMMARY

A detailed examination of the urinary steroids was made in 13 women with hirsutism, including 4 with associated clinical and pathological findings typical of the Stein-Leventhal syndrome. Excessive production of two adrenal hormone metabolites, androsterone and etiocholanolone, was noted in all subjects together with a lesser but significant elevation in the metabolites of 11-oxygenated hormones.

In the light of previous knowledge concerning the source and physiologic character of the hormonal precursors of these metabolites, it can be presumed that the steroidal aberration manifest in these women was a relative hypersecretion of androgenic hormone, or hormones, from the adrenal cortex. A significant relationship is strongly implied between the excess production of adrenal androgen and the hypertrichosis in these women.

[3] Since this manuscript was submitted, Perloff, Hadd, Channick, and Nodine (23) have reported studies of steroid excretion by hirsute women similar to those described in the present communication. Their conclusions confirm the interpretation given to an earlier study (3) and extend the experimental findings to a larger group of women. Their results and conclusions, considered with the present report, leave little doubt that "idiopathic" hirsutism is more properly categorized as a type of adrenal hyperfunction.

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SALICYLATE AND SALICYL CONJUGATES: FLUORIMETRIC ESTIMATION, BIOSYNTHESIS AND RENAL EXCRETION IN MAN[1]

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Three urinary salicyl conjugates account for over 80 per cent of salicylate ingested by a normal subject excreting acid urine (1–4). The metabolites, which are estimated by specific methods, are salicylurate, salicyl *acyl* glucuronide, and salicyl *phenolic* glucuronide (1). Alkaline urines also contain the same conjugates plus variable amounts of salicylate (5–8). With the existing methods, only salicylate has been detected in human plasma (9). It has been suggested, therefore, that the salicyl conjugates are formed within the kidney in man (7).

The present report describes sensitive, fluorimetric methods for the specific estimation of salicylate and each of its principal metabolites in plasma and urine. With these methods, the salicyl conjugates have been detected and estimated in the plasma of normal subjects given a single oral dose of salicylate and values for the renal clearance of each compound obtained. The clearances do not appear to exceed the renal plasma flow, suggesting that salicylate conjugation is mainly extrarenal in man. In addition, the renal clearance values yield information concerning the mechanisms for the urinary excretion of conjugated and unconjugated salicylate.

METHODS AND MATERIALS

Drug experiments. The experiments were conducted in 21 normal male subjects, aged 14 to 63, including 17 medical students, resident physicians, and technicians, aged 22 to 35. The group included 17 white subjects, 3 Negroes, and 1 Chinese. During the experiments the subjects drank sufficient water to maintain urine flows greater than 1 ml. per minute; otherwise their diets and activities were unrestricted.

Sodium salicylate powder, 0.4 millimole per kilogram body weight to a maximal dose of 4.0 grams, was given in one oral dose, either in solution or in gelatin capsules. Samples of blood and urine were collected for as long as 30 hours after the drug. Voided urine samples were collected for periods of 90 to 120 minutes, with a minimum of two successive collection periods. Venous blood samples, drawn with oxalate at the mid-points of the collection periods, were centrifuged at once at 5° C. for 15 minutes. All plasma and urine samples were treated immediately with neutral hydroxylamine to convert the unstable salicyl acyl glucuronide (SAG) to its stable hydroxamate derivative (1). Plasma and urine samples obtained prior to the drug were found to contain minimal quantities of fluorescent materials, as estimated by the following methods.

Fluorimetric estimation of plasma salicyl compounds. The fluorescence of sodium salicylate and salicylurate was measured with the Aminco-Bowman spectrophotofluorometer (10). In neutral aqueous solutions, salicylate fluorescence exhibited its peak intensity at 420 mμ, when activated at the optimal wave length, 310 mμ. As little as 1.0 μM per liter (10^{-6} M) salicylate can be detected, and the intensity of fluorescence is proportional to the salicylate concentration. The salicylate fluorescence remains constant from pH 5 to pH 12. In contrast, the fluorescence of salicylurate solutions increases fortyfold as the pH increases from 6 to 10, and remains maximal thereafter (11). This increment in fluorescent light (peak intensity 420 mμ, activated at 340 mμ) is proportional to the salicylurate concentration, and independent of the salicylate concentration. Each of the salicyl conjugates can be estimated in plasma in a concentration as little as 1.0, μM per liter, and in the presence of a one thousandfold excess of salicylate.

Salicyl acyl glucuronide. Five ml. of plasma is incubated with 4.0 ml. of 2 M hydroxylamine at pH 7.0 for two hours at room temperature to convert SAG to salicyl hydroxamate, and the latter is extracted into 30.0 ml. of ether by shaking for two minutes. A 20 ml. aliquot of ether is washed successively with 4.0 and 2.0 ml. of 0.4 M sodium phosphate of pH 7 to remove traces of salicylate. Salicyl hydroxamate solutions exhibit very little fluorescence, but on acid hydrolysis they liberate salicylic acid, which can be estimated fluorimetrically. Accordingly, duplicate aliquots of the washed ether are evaporated to dryness at 30 to 40° C. One of the samples is hydrolyzed in 6 N H_2SO_4 at 100° C. for 30 minutes. Subsequently, both samples are adjusted to pH 7.4 with 0.2 M sodium phosphate buffer. The increment in fluorescence (activating wave length 310 mμ, fluorescence wave length

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420 mμ) following hydrolysis is proportional to the quantity of salicyl hydroxamate added. Because pure SAG was not available, a urine sample containing the glucuronide in high concentration, and standardized against crystalline salicyl hydroxamate, was the reference standard. Recoveries of the urinary SAG varied from 86 to 114 per cent, and were determined with each series of determinations.

Salicylate and salicylurate. After extraction of salicyl hydroxamate, the residual plasma is diluted 1:2 and the protein is precipitated by the addition of perchloric acid to a final concentration of 7 per cent. After centrifugation, 10.0 ml. of the supernatant is shaken for two minutes with 15.0 ml. of ether to extract salicylic and salicyluric acids. One ml. of the ether is evaporated to dryness, the dried residue dissolved in 0.2 M sodium phosphate pH 7.4, and the salicylate, present in great excess relative to salicylurate, estimated fluorimetrically. Ten ml. of ether is evaporated to dryness, the residue dissolved in 4.0 ml. of 0.05 N H_2SO_4, and the resulting mixture of salicyluric and salicylic acids washed twice with nine volumes of water-saturated CCl_4 to remove salicylic acid (5). Salicyluric acid is extracted from the CCl_4-washed solution with two volumes of ether, and duplicate portions of the ether evaporated to dryness. One dried residue is dissolved in 0.2 M sodium phosphate (pH 6.0), the other in 0.5 M Na_2CO_3 (pH > 10.5), and the salicylurate concentration estimated by the increment in fluorescence at the alkaline pH (activating wave length 340 mμ, fluorescence wave length 420 mμ). The recoveries from plasma of added salicylate and salicylurate varied from 80 to 90 per cent, and were determined with each series of estimations.

Salicyl phenolic glucuronide. Residual traces of salicylic acid are removed from the protein-free supernatant by washing twice more with one and one-half volumes of ether. When a portion of the washed solution is subsequently hydrolyzed in 6 N H_2SO_4 at 100° C. for 90 minutes, salicyl phenolic glucuronide (SPG) liberates an equimolar quantity of salicylic acid. The latter is extracted with an equal volume of ether and estimated fluorimetrically as described. A duplicate, unhydrolyzed aliquot serves as the control. When a sample of urine containing SPG in high concentration was added to plasma, the recoveries varied from 60 to 65 per cent, and were determined with each series of estimations.

Fluorimetric estimation of urinary salicyl compounds. The methods for urine are modifications of those for plasma. After incubation of 1.0 ml. of urine with 1.0 ml. of neutral hydroxylamine, the salicyl hydroxamate formed is extracted with 10.0 ml. of ether. Portions of the ether are evaporated to dryness and the salicylic acid released on acid hydrolysis is determined fluorimetrically. When the urine is alkaline and contains excess salicylate, 8.0 ml. of ether is washed with 1.0 ml. of 0.2 M sodium phosphate, pH 7.4, before evaporation and hydrolysis. The SAG-free urine is acidified to pH 2 with H_2SO_4 and extracted with two volumes of ether to remove salicylic and salicyluric acids. A portion of the ether is evaporated to dryness, the residue is dissolved in 1.5 ml. of 0.05 N H_2SO_4, and equilibrated with 10.0 ml. of water-saturated CCl_4. One portion of the aqueous layer containing the salicyluric acid is adjusted to pH 6 with 0.2 M sodium phosphate and another to pH > 10.5 with 0.5 M Na_2CO_3. Salicylurate is estimated by the increment in fluorescence observed at the alkaline pH. Salicylate is extracted from an aliquot of the CCl_4 layer with five volumes of 0.2 M sodium phosphate, pH 7.4, and estimated fluorimetrically. Finally, the ether-extracted urine is washed twice more with equal volumes of ether, and SPG is estimated by the salicylic acid liberated on acid hydrolysis. Crystalline salicyl hydroxamate, salicylate and salicylurate were the reference standards for these determinations.

Binding of salicyl compounds to plasma proteins. Information concerning the binding of salicylate and its principal conjugates on the plasma proteins was obtained for use in the interpretation of the clearance data. The protein binding of each salicyl compound in whole plasma was studied by the dialysis-equilibrium technique, using cellophane (Visking) membranes.[2] The data obtained with all four compounds conformed to the Freundlich isotherm ($x/m = Kc^{1/n}$), with the range of concentrations examined and a plasma albumin of 2.8 grams per cent. The binding constants obtained were as follows: for salicylate (at plasma levels of 580 to 1,810 μM per liter) K equals 8.03, 1/n equals 0.35; for salicylurate (at plasma levels of 11 to 223 μM per liter) K equals 0.69, 1/n equals 0.77; for SPG (at plasma levels of 6 to 95 μM per liter) K equals 0.16, 1/n equals 0.72; and for SAG (at plasma levels of 4 to 64 μM per liter) K equals 0.03, 1/n equals 0.76. The filtrable fraction (F) of each compound was obtained

[2] We are grateful to Dr. J. V. Taggart for details of the following unpublished method. Two ml. plasma is introduced into a bag of 1.5 inch cellophane (Visking) sealed at the bottom by glass rods held together with rubber bands. The upper end is similarly sealed, the bag placed flat in a Petri dish, and covered with 20.0 ml. of 0.067 M sodium phosphate, pH 7.4, containing 0.05 M NaCl. The covered Petri dish is rotated 80 times per minute on a moving platform. Dialysis equilibrium is achieved within four hours, when the compound studied is added either to the plasma or to the dialyzing medium. A specimen of blood bank plasma was used for studies with the salicyl compounds. The sources of the salicyl glucuronides were the urine standards for plasma estimations. Varying amounts of the salicyl compound were added to the plasma and the dialyzing medium to approximate the equilibrium concentrations. After rotation at 5° C. (to minimize hydrolysis of the unstable SAG) for 6 hours, 85 to 95 per cent of the added salicyl compounds were recovered. The protein binding conformed in each instance to the Freundlich isotherm: $x/m = Kc^{1/n}$, where x is the number of μM of salicyl compound bound by m grams of albumin, c is the equilibrium concentration of free salicyl compound in plasma water (μM per liter), and k and 1/n are constants.

TABLE I

Plasma concentrations of salicyl compounds after one oral dose of salicylate

Hours after ingestion	Number of subjects	Plasma concentration*			
		Salicylate	Salicylurate	SAG†	SPG‡
2	18	1,782§ ± 245	9.8 ± 1.9	6.8 ± 2.1	7.7 ± 3.5
4	19	1,628 ± 179	10.1 ± 2.3	6.2 ± 1.7	12.5 ± 4.1
8	7	1,400 ± 110	9.5 ± 2.0	4.3 ± 0.7	16.4 ± 2.3
24	9	467 ± 200	10.5 ± 2.2	2.2 ± 1.1	14.8 ± 4.6

* Concentration in μM per liter plasma, expressed as the mean plus or minus one standard deviation.
† Salicyl acyl glucuronide.
‡ Salicyl phenolic glucuronide.
§ Corresponds to 24.6 mg. per cent.

from the isotherm.[3] In three experiments (Figure 3) the filtrable fraction of salicylate was determined directly by dialysis of plasma samples at 38° C. The values observed were 0.08 to 0.12, and corresponded to the values obtained from the isotherm.

The renal clearance (ml. per minute) of "free," or nonprotein bound, salicylate (C_{FrS}) was calculated as $C_{FrS} = UV/PF$, where U and P are the salicylate concentrations (μM per liter) in urine and plasma, V is the urine flow (ml. per minute), and F is the filtrable fraction. A similar expression was used to calculate the clearance of "free" SPG.

Other methods and materials.[4] Urine pH values were determined with a Beckman glass electrode pH meter immediately after voiding. The endogenous creatinine clearance served as an estimate of the glomerular filtration rate (15). Total plasma protein concentration was estimated by the biuret method (16), and the plasma albumin level with the Spinco Model R paper electrophoresis apparatus. Optical density measurements were made in the Beckman DU spectrophotometer. The preparation of crystalline salicyluric and salicyl hydroxamic acids has been described (1).

[3] F is defined as the ratio c/P, where c is the concentration of the freely diffusible compound in plasma water, and P is the plasma concentration (12). From the Freundlich isotherm c, and hence F, may be calculated when P, the constants for the isotherm, and the plasma albumin (estimated electrophoretically) are known.

[4] A conjugate of salicylurate appears in the plasma and urine of normal subjects after ingestion of salicylate. This material, which may be identical with "uraminosalicylsaüre" (3), cannot be extracted with ether at pH 2. After hydrolysis of an ether-washed solution in 6 N H_2SO_4 for 90 minutes at 100° C., salicyluric acid is liberated and estimated fluorimetrically (plasma) or with a paper chromatographic method (urine), using the colorimetric reaction of Gaffney, Schreier, DiFerrante, and Altman (13) to estimate the salicylurate areas on the chromatogram (1, 14). The plasma levels and excretion rates of this metabolite increased for 24 hours following oral salicylate, and reached values of 3.5 to 5.5 μM per liter and 0.2 to 0.6 μM per minute, respectively.

RESULTS

Salicyl compounds in plasma

After a single oral dose of salicylate, detectable quantities of salicylurate, SAG and SPG appeared in the plasma of each subject. The mean concentrations observed at various times following the administration of salicylate are listed in Table I. The time course of each salicyl compound in plasma is illustrated in Figure 1. The patterns of the curves were similar for all the subjects. Maximal plasma concentrations of salicylate, salicylurate and SAG were observed within two hours after salicylate, when mean levels of 1,780, 9.8 and 6.8 μM per liter, respectively, were attained. The maximal concentrations of SPG were observed only after eight hours and reached 16.4 μM per liter. The levels of salicylate and SAG fell progressively, while those of salicylurate and SPG remained fairly constant for 24 hours

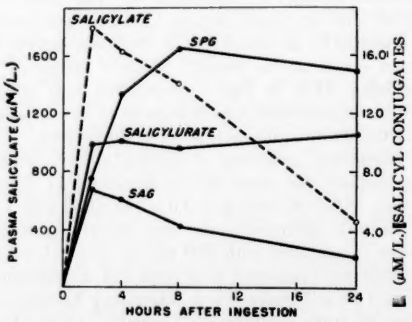

FIG. 1. MEAN PLASMA CONCENTRATIONS OF SALICYLATE (--○--) AND SALICYL CONJUGATES (—●—) AFTER ONE ORAL DOSE OF SALICYLATE

The right- and left-hand vertical scales refer to the salicyl conjugates and to salicylate, respectively.

Salicylate was the principal salicyl compound in plasma, with a concentration 70 times that of the combined conjugates 2 hours after the drug, and 15 times that of the conjugates after 24 hours.

Salicyl compounds in urine

The maximal urinary excretion rates of the salicyl conjugates, listed in Table II, reflect the capacity of normal subjects to form and excrete these compounds. Salicylate is conjugated slowly in man. For example, the maximal rate of excretion of salicylurate in the present studies was approximately 5 μM per minute. After a comparable dose of benzoate, the hippurate excretion can exceed 100 μM per minute (1, 17). After ingestion of *p*-methoxybenzoate, the rate of acyl glucuronide excretion can be six times that of SAG; and after salicylamide the phenolic glucuronide excretion can be 20 times that of SPG (18).

When the excretion of total salicyl conjugates was maximal, two to six hours after the drug, salicylurate accounted for 64 per cent, SAG for 23 per cent, and SPG for 13 per cent of the urinary conjugates. These values agree with previous observations (1–3). The rate of excretion of each conjugate remained approximately proportional to its plasma concentration. As previously reported (1), SAG was the principal glucuronide in the early hours following the drug, and SPG predominated in the later hours.

Water diuresis, with increases in urine flow to 12 ml. per minute, failed to alter significantly the excretion rates of the conjugates. Moreover, the rates were unchanged in three subjects when the oral administration of 10 grams of sodium bicarbonate increased the urine pH from an original level of 5.0 to 6.2 to a final value of 7.6 to 8.0. In contrast, bicarbonate administration increased the excretion of salicylate from approximately 1.5 μM per minute to 12 μM per minute. Thus, the total salicyl excreted at any given time includes a relatively predictable quantity of salicylate conjugates and a variable, pH-dependent quantity of salicylate.

TABLE II

Maximal rates of urinary excretion of salicyl conjugates after one oral dose of salicylate

Metabolite	Number of subjects	Hours after ingestion*	Maximal rate of urinary excretion†
Salicylurate	19	2–6	5.2 ± 1.1
SAG‡	19	2–6	1.9 ± 0.4
SPG‡	6	6–10	1.3 ± 0.2
Total salicyl glucuronides	19	2–6	3.0 ± 0.6
Total salicyl conjugates	19	2–6	8.1 ± 1.1

* The period during which the maximal rate of excretion was observed.

† Micromoles per minute; mean plus or minus one standard deviation.

‡ SAG, salicyl acyl glucuronide; SPG, salicyl phenolic glucuronide.

Renal clearances of salicyl conjugates

The renal clearances of salicylurate, SAG and "free" SPG considerably exceeded the endogenous creatinine clearances, as summarized in Table III. Thus glomerular filtration and tubular secretion appear to be the principal renal mechanisms for the excretion of these compounds. The clearances of the salicyl conjugates did not exceed the approximate renal plasma flows, when the latter were calculated from the endogenous creatinine clearances and a normal value (0.19) for the filtration fraction in man (12). Therefore, no evidence for renal synthesis of the conjugates was obtained.

TABLE III

Renal clearances of endogenously formed salicyl conjugates

Metabolite	Plasma concentration	Number of clearance periods	Number of subjects	Renal clearance*	Renal clearance/ endogenous creatinine clearance
	μM/L.			*ml./min.*	
Salicylurate	5.7–14.9	70	19	444 ± 112	4.2 ± 0.9
SAG†	3.0– 6.0	29	13	331 ± 70	3.3 ± 0.8
SPG†	10.0–15.0	22	12	73 ± 22	0.7 ± 0.2
"Free" SPG‡	2.0– 3.0	22	12	384 ± 145	3.0 ± 1.0

* Each clearance value has been adjusted to 1.73 square meters body surface, and is expressed as the mean plus or minus one standard deviation.

† SAG, salicyl acyl glucuronide; SPG, salicyl phenolic glucuronide.

‡ Nonprotein bound SPG.

Clearance studies with salicylurate by Smith, Finkelstein, Aliminosa, Crawford, and Graber (19) in two male subjects yielded salicylurate to inulin clearance ratios of 2.9 and 4.3. These figures agree with the clearance data obtained in the present study with endogenously formed salicylurate, and suggest that the renal synthesis of salicylurate is relatively unimportant.

The effect of probenecid on the salicyl conjugate clearances was studied in six subjects. After two control periods, 2.0 grams of probenecid was given by mouth and the effects observed for five hours. Probenecid, an inhibitor of certain tubular secretory mechanisms (20), uniformly reduced the salicylurate clearances by 29 to 53 per cent (mean, 40), and the SAG clearances by 42 to 71 per cent (mean, 57). There was a concomitant rise in the plasma levels of these metabolites. The SPG clearances were altered less consistently, with a fall of 42, 43 and 46 per cent in three subjects, and no significant change in three others.

The plasma concentration of SAG appeared to influence its renal clearance. With plasma levels of 1 to 3, 3 to 6, and 6 to 12 μM per liter, the corresponding clearance rates (ml. per minute) were 502 ± 84 (16 periods, 11 subjects),[5] 331 ± 70 (29 periods, 13 subjects), and 254 ± 27 (18 periods, 10 subjects). A similar but less marked trend was noted for the SPG clearances. The loads of SAG and SPG presented to the renal tubules (μM per minute), and the rates of secretion of the glucuronides were calculated by conventional methods[6] (12), and plotted in Figure 2. At comparable loads, the SAG secretion considerably exceeded that of SPG, and with loads less than 1.0 μM per minute SAG secretion was essentially complete. Secretion of both glucuronides became progressively less complete at higher loads, suggesting a limitation in the capacity of the tubules to transport these compounds.

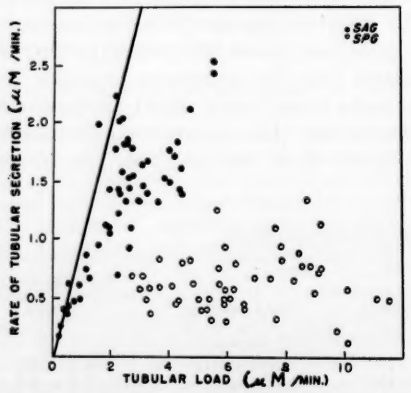

FIG. 2. RATE OF TUBULAR SECRETION OF SALICYL GLUCURONIDES AT VARIOUS TUBULAR LOADS

The straight line connects the points at which the tubular load is completely secreted.

[5] Mean plus or minus one standard deviation.

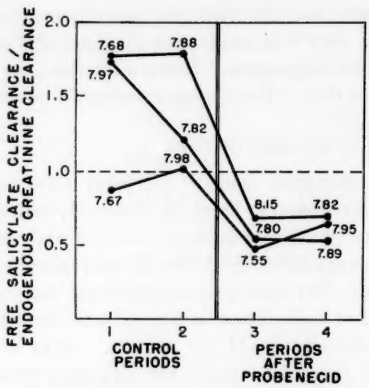

FIG. 3. EFFECT OF PROBENECID ON THE "FREE" SALICYLATE TO ENDOGENOUS CREATININE CLEARANCE RATIO

The lines connect the values for an individual subject. The urine pH value for each period is indicated.

Variations in urine flow from 1 to 12 ml. per minute, or in urine pH from 6 to 8, did not significantly affect the clearances of the salicyl conjugates.

Renal clearances of salicylate

Alkalinization of the urine causes a marked increase in the clearance of "free" salicylate (C_{FrS}), as described previously (5–8), and confirmed in the present studies with methods which clearly distinguish between conjugated and unconjugated salicylate. When urine pH was less than 6, C_{FrS} was 5 to 15 per cent of the endogenous creatinine clearance, indicating that glomerular filtration and

[6] Tubular load of $x = P_xRPF - P_xC_FFW$, where P_x is the plasma concentration, RPF is the renal plasma flow, C_F is the glomerular filtration rate, F is the filtrable fraction, and W is the plasma water content expressed as a fraction. Tubular secretion rate of $x = U_xV - P_xC_FFW$, where U_xV is the total rate of excretion of x. C_F was estimated by the endogenous creatinine clearance, and RPF was calculated at $C_F/0.19$.

tubular reabsorption were the renal mechanisms involved. With urine pH greater than 7.4, C_{FrS} to endogenous creatinine clearance ratios exceeded 1.0 in 4 of 7 subjects (9 of 15 clearance periods), and the highest value attained was 1.8 (Figure 3). Previous investigators have reported values greater than 1.0 for the salicylate clearance to filtration rate ratio in man (5, 7, 8), the dog (21), and the rabbit (22). These observations suggest that tubular secretion is a third mechanism involved in salicylate excretion.

It has been proposed that tubular transport of salicylate, either reabsorptive or secretory, could result from a passive transfer process, dependent on a pH gradient between the tubular urine and the peritubular fluid (23). To investigate this possibility, the following experiment was performed in three subjects. The urine pH was maintained consistently greater than 7.5 for the experimental period of nine hours by the oral administration of 18 grams of sodium bicarbonate in divided doses. C_{FrS} in two subjects exceeded the endogenous creatinine clearances, as shown in Figure 3. The subjects then ingested 2.0 grams of probenecid. A fall in C_{FrS} was observed in each instance, with no significant change in urine pH, endogenous creatinine clearance, or the fraction of salicylate bound to plasma proteins. Gutman, Yü, and Sirota (8) have also observed a reduction in C_{FrS} after probenecid. These results provide supportive evidence for the tubular secretion of salicylate, for it seems highly improbable that probenecid would enhance salicylate reabsorption. Inasmuch as probenecid can inhibit tubular transfer processes, presumably by a direct action on the cells, the secretion of salicylate appears to require cellular activity. The C_{FrS} to endogenous creatinine clearance ratios following probenecid varied from 0.5 to 0.7 (Figure 3), with urine pH values close to 8. Thus the tubules can reabsorb salicylate at a time when a highly alkaline urine is being elaborated. These findings are not consistent with the view that only pH gradients across the tubule are responsible for the transport of salicylate.

DISCUSSION

Metabolism of salicylate

The methods developed for the estimation of salicyl conjugates in the plasma, as well as in the urine, have permitted more detailed observations on the fate of salicylate in normal men. Following an oral dose, the drug is rapidly absorbed and slowly conjugated with glycine or glucuronic acid, mainly at extrarenal sites. The conjugates are excreted in part by tubular secretion, and the renal clearance of these metabolites considerably exceeds that of salicylate. Consequently, the bulk of the urinary salicyl may be in the form of conjugates, while the principal compound in the plasma is salicylate. The conjugation of other compounds has also been observed to result in products which are cleared by the kidneys more rapidly than is the parent compound (24–26).

The rates of urinary excretion of the salicyl conjugates are related to their rates of net synthesis in the body. SAG is formed relatively rapidly for two hours after oral salicylate. In this time the urinary excretion of SAG exceeds that of SPG by as much as fourfold in some subjects. Although the plasma levels of the two glucuronides are approximately equal at two hours (Figure 1), SAG excretion exceeds that of SPG because of its greater rate of clearance. A progressive reduction in net synthesis of SAG is noted as the plasma salicylate concentration falls. In contrast, a fairly constant synthesis of SPG and salicylurate, or a slow release of the compounds from the tissues, is maintained for as long as 24 hours after the drug is administered.

The organs which conjugate salicylate in man are unknown. By analogy with the probable formation of hippurate by human liver (27), and the synthesis of bilirubin glucuronide by human liver homogenates (28, 29), it seems probable that this organ is a major site for similar conjugations. Recently, evidence has been obtained in this laboratory for the *in vitro* synthesis of salicyl glucuronides by slices of intestinal mucosa, kidney, liver, spleen, lung and urinary bladder from various animal species.[7] It is possible, therefore, that the glucuronides may be formed by many human tissues.

[7] The formation of salicyl glucuronides *in vitro* was observed with the following tissues: intestinal mucosa (guinea pig, rabbit, hamster, rat, cat); liver (guinea pig, rat, hamster, dog); kidney (guinea pig, rabbit, rat, hamster, dog); urinary bladder (guinea pig, rabbit); spleen (guinea pig, hamster); and lung (guinea pig).

Renal clearance of salicylate

In the present studies salicylate was estimated with a relatively specific method. The clearance data obtained confirm and extend the observations of previous investigators (5, 7, 30). Salicylate, like thiosulfate (31) and potassium ion (32, 33), can be reabsorbed and secreted by the renal tubules, as well as filtered at the glomerulus. Tubular reabsorption predominates when the urine is acid. Tubular secretion is evident after alkalinization of the urine, which appears to partially inhibit the reabsorptive process. Probenecid causes a reduction in salicylate clearance, which is not dependent on a change in urine pH, and is probably due to inhibition of active tubular secretion.

It is of interest that low doses of salicylate, probenecid and other uricosuric drugs may depress the renal clearance of urate in man, as demonstrated by Yü and Gutman (34). The evidence for active tubular secretion of salicylate, which can be inhibited by probenecid, lends support to a suggestion by Yü and Gutman that urate, salicylate and other substances may compete for a common tubular secretory mechanism.

SUMMARY

1. Fluorimetric methods are described for the detection and estimation of salicylate, salicylurate, salicyl acyl glucuronide, and salicyl phenolic glucuronide in plasma and urine.

2. Salicylate conjugation is mainly extrarenal in man. The conjugates formed are secreted by the renal tubules, and their clearance exceeds that of salicylate.

3. The net synthesis of salicyl acyl glucuronide generally exceeds that of salicyl phenolic glucuronide immediately after the oral administration of salicylate, and the converse is observed 8 to 24 hours later. The renal tubules secrete the acyl glucuronide more rapidly than the phenolic glucuronide.

4. The renal excretion of unconjugated salicylate involves glomerular filtration, tubular reabsorption, and active tubular secretion.

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STUDIES ON THE CAUSAL ROLE OF HYPOALBUMINEMIA IN EXPERIMENTAL NEPHROTIC HYPERLIPEMIA [1]

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Previous work on the pathogenesis of the nephrotic hyperlipemia, in rats injected with anti-rat kidney sera obtained from rabbits (nephrotoxic serum, NTS), has indicated that the increase in plasma lipid concentration precedes the development of hypoproteinemia (1). They furthermore have shown that the thyroid, pituitary, adrenals, ovaries, testes and pancreas glands are not essential for its elicitation (2); that subtotal hepatectomy inhibits its development (3); and that in contradistinction to other fat depots, the hepatic lipid values are decreased (4). It was also shown that the metabolic utilization of C^{14} labeled trilaurin and the clearance of this substance from the blood stream after intravenous injection proceeded at normal rates (5). It was finally observed that renal tissue is essential for its elicitation, and that the severity of the hyperlipemia is related to the amount of present nephrotic kidney tissue (6).

Recently Rosenman, Friedman, and Byers have stated (7) that "the hyperlipemia observed in the experimental nephrotic syndrome is initiated and maintained by the external renal loss of plasma albumin."

The observations reported in the following studies were stimulated by the work of these authors. They are concerned with: a) relation of severity of hyperlipemia to severity of hypoalbuminemia; b) time relationship in the development of hyperlipemia and hypoalbuminemia; and c) the effect of saline, protein-free control urine, nephrotic urine, and dextran solutions on serum cholesterol and total lipid concentrations in nephrotic rats.

METHODS AND PROCEDURES

Rats of the Sprague-Dawley strain were used. They were fed Friskies and housed in single cages. Methods used to produce the nephrotic syndrome in rats and to determine the chemical data reported in this study have been described previously (8–12). Blood for analysis was obtained after food had been withheld for 16 hours.

A first group of studies was designed to establish a possible relationship between severity of hypoalbuminemia and hyperlipemia in the experimental nephrotic syndrome. Serum protein, serum albumin, and total lipid values were obtained in 19 nephrotic rats of our colony in whom nephrotic renal disease had persisted uninterruptedly for 1 to 11 months. These animals had been studied with weekly quantitative urinary protein determinations and monthly blood pressure readings. Their food intake was normal and their weight curves were indistinguishable from control animals.

A second group of studies was planned to investigate the time relationship in the development of hypoalbuminemia and hyperlipemia within the first 24 hours after injection of NTS. Twenty-six rats of the same weight and sex were used for this purpose. They were given NTS by intravenous injection. The potency of the two batches of pooled sera used in these experiments had been previously established by bio-assay. In amounts of 0.69 and 1.38 ml. per 100 Gm. rat, they regularly induced nephrotic renal disease of equal severity, inducing proteinuria and hyperlipemia within the first 24 hours. The animals were killed 2, 4, 6, 8 and 24 hours after injection. It was essential to obtain these values from the 26 separate animals killed in the quoted intervals after the injection of NTS, because it had been noted that in rats the removal of 3 to 4 ml. of blood alone may decrease blood lipid and cholesterol values appreciably for 3 to 5 days.

Rosenman, Friedman, and Byers (7) have shown that when the loss of urinary proteins was prevented by ligation of the ureter on one side and lower vena cava-ureter anastomosis on the other side in rats injected with NTS, the development of hyperlipemia was inhibited. The intravenous infusion of bovine albumin acted similarly and diminished the degree of hyperlipemia in rats with established nephrotic renal disease. It seemed necessary to us to control these experiments by studying the effect of nonprotein-containing rat urine, and also to investigate the effect of dextran instead of albumin on the hyperlipemia of nephrotic rats. For this reason, experiments were planned to study the effect of bleeding, injections of saline, control and nephrotic urines, and dextran solutions [2] on the nephrotic hyperlipemia (Table I). Forty-

[1] Aided by grants of the Cleveland Area Heart Society and the Northern Ohio Chapter of the National Nephrosis Foundation.

[2] Plavolex®, Wyeth (6 per cent dextran in 0.9 per cent NaCl solution).

three nonedematous nephrotic rats were used. These had been injected with antikidney serum one and one-half to seven months prior to the first bleeding. They had uninterrupted proteinuria (100 to 400 mg. per 24 hours), ate well, gained normally in weight, and all had marked hyperlipemia with values ranging for total lipids from 1,050 to 2,300 mg. per cent and for cholesterol from 160 to 420 mg. per cent. For reasons quoted above bleeding was spaced six to eight days apart. Initial values were obtained three days prior to the onset of treatment, and 3.5 to 4 ml. of blood was obtained from the infraorbital sinus (13) every six to eight days thereafter for three to four weeks. All solutions were given by intraperitoneal injections for 10 consecutive days in amounts of 4 ml. twice daily. Nephrotic and protein-free control urines were collected after 10 mg. of streptomycin and 10 mg. of tetracycline (Achromycin®) per 20 ml. of urine had been added to the sterilized recipient jar. The samples were then filtered through a No. 6 Seitz filter and urinary cultures were obtained to assure sterility.

RESULTS

Figure 1 represents serum protein and serum albumin values (ordinate) related to the concentration of serum total lipids (abscissa) obtained in 19 nephrotic rats with well-established disease of 1 to 11 months' duration. It is evident from these results that in these animals there is no correlation between severity of hypoproteinemia or hypoalbuminemia and severity of hyperlipemia.

Figure 2 represents serum total lipid, cholesterol, serum protein and albumin values obtained in 26 rats 2, 4, 6, 8 and 24 hours after the intravenous injection of antikidney sera obtained from rabbits. From the 30 rats originally injected, 4 rats were killed accidentally and were not available for bleeding after 8 and 24 hours. It can be seen (Figure 2) that total lipid and cholesterol values were increased beyond values ever obtained in control rats in some instances two hours, in most instances four hours, and in all instances eight hours after the injection of NTS. Serum albumin concentration, however, remained within the range of normal without exception for six hours after NTS injection. After eight hours, when hyperlipemia was established in all instances, only one of four albumin values was decreased below normal range.

Table I summarizes the results obtained in 43 nonedematous nephrotic rats which were bled every six to eight days without being subjected to any intraperitoneal injections (eight rats, column

TABLE I

Effect of bleeding, intraperitoneal injections of saline, control and nephrotic urines, and dextran solutions on serum protein, blood urea nitrogen (BUN), serum cholesterol and total lipids *

Rat no.	Bleeding only				Saline				Control urines				Nephrotic urines				Dextran solutions			
	S.P.†	BUN†	Chol.†	T.L.†	S.P.	BUN	Chol.	T.L.	S.P.	BUN	Chol.	T.L.	S.P.	BUN	Chol.	T.L.	S.P.	BUN	Chol.	T.L.
1	−	−	−	−	−	+	−	−	−	+	−	+	−	−	+	+	+	+	+	+
2	−	−	−	−	−	+	−	−	−	−	−	+	−	−	−	+	+	+	+	+
3	−	−	−	−	−	−	−	−	−	−	+	+	−	−	+	+	+	+	+	+
4	−	−	−	−	−	−	−	+	−	−	−	+	−	+	+	+	+	+	+	+
5	−	−	−	−	−	+	−	+	−	−	−	+	−	+	+	+	+	+	+	+
6	−	−	−	−	−	−	−	−	−	−	+	+	−	−	+	+	+	−	+	+
7	−	−	−	−	−	−	−	+	−	−	−	−	−	−	+	+	+	−	+	+
8	−	−	−	−	−	+	−	−	−	−	+	+	−	+	+	+	+	−	−	+
9																	−	+	+	+
10																	−	−	−	+
11																	+			+

* A decrease of these values during treatment and their return to pretreatment levels within two weeks after treatment had been discontinued is charted as follows: A decrease within 10 per cent of the original values is recorded as −. Decreases in excess of 30, 50 and 70 per cent are recorded as +, ++ and +++, respectively.

† S.P., serum protein; BUN, blood urea nitrogen; Chol., serum cholesterol; T.L., total lipids.

1); rats which were bled in identical intervals and were given 4 ml. of saline twice daily for 10 days (eight rats, column 2); rats which received 4 ml. of protein-free urine twice daily (eight rats, column 3); protein-containing nephrotic urine (eight rats, column 4); and dextran solution (11 rats, column 5) by intraperitoneal injections. If serum protein, blood urea nitrogen, cholesterol or total lipid values decreased to 30 to 50 per cent of the original value obtained three days prior to the onset of treatment, and if they returned to pretreatment levels within two weeks after treatment had been discontinued, the result is listed in Table I as +. If the decrease amounted to 50 to 70 per cent, it is registered as ++, and in excess of 70 per cent as +++.

It can be seen (Table I, column 1) that the removal of 3 to 4 ml. of blood every six to eight days does not alter the concentration of any of the serum constituents examined. Considering cholesterol and total lipid values, it can be noted (Table I, column 2) that total lipid values decrease temporarily only twice out of eight times during the 10 day period of saline administration, whereas the cholesterol concentration was not affected. The intraperitoneal injection of 8 ml. of protein-free control urine for 10 days decreased the elevated cholesterol values three times and the hyperlipemic total lipid values six times of eight such experiments. The magnitude of this effect was the same as when protein-containing nephrotic urine was used (Table I, column 4). The only difference was that in nephrotic rats treated with nephrotic urines, the concentration of total lipids decreased in each of the eight experiments and the cholesterol values seven out of eight times. When dextran solutions were used, the temporary decrease of lipid and cholesterol was as regularly and markedly noted as in the studies using protein-containing nephrotic urines. Because of the osmotic activity of dextran solutions, hematocrit readings were obtained at each bleeding in 3 of the 11 rats. Even though an initial decrease from 46, 37 and 43, respectively, to 30 was noted in each instance, no correlation with total lipid or cholesterol concentration was noted. While the decreased hematocrit readings did not return to normal during the period of observation, the cholesterol and total lipid values increased to the pretreatment levels within a week after the administration of dextran solutions had been discontinued. It should be re-emphasized that none of the nephrotic rats used in these studies was in the edematous phase of the disease.

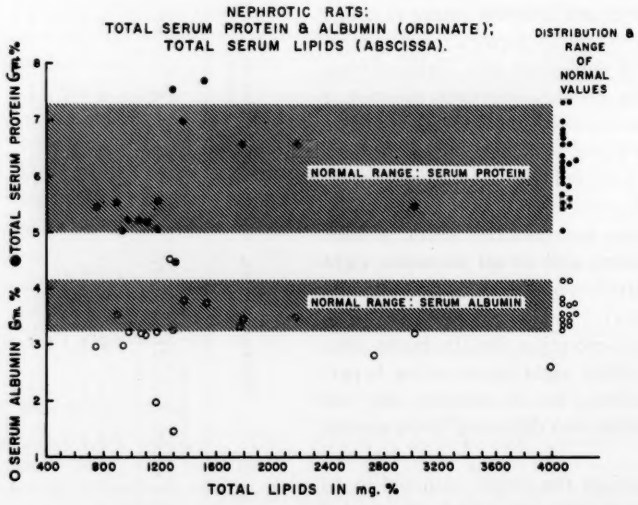

FIG. 1. CORRELATION OF SERUM PROTEIN AND SERUM ALBUMIN CONCENTRATION (ORDINATE) WITH TOTAL LIPID VALUES (ABSCISSA) IN SERA OF 19 NEPHROTIC RATS

DISCUSSION

If hypoalbuminemia were the only cause for the nephrotic hyperlipemia, an inverse relationship between severity of plasma albumin depletion and severity of the hyperlipemia could have been expected. That this has not been established (Figure 1) does not, however, eliminate the hypoalbuminemia as one of several factors that may pathogenetically be at play in the elicitation of the nephrotic hyperlipemia.

It has furthermore been shown (Figure 2) that hypoalbuminemia does not precede the development of hyperlipemia if studies are carried out within the first two to four hours after the injection of NTS. It can, in fact, be seen (Figure 2) that hypoalbuminemia succeeds the development of the hyperlipemia in most instances. If hyperlipemia can be noted within two hours after the administration of NTS it is almost inconceivable that hypoalbuminemia could have developed prior to that period of time.

The studies summarized in Table I show that the concentration of serum total lipids and cholesterol can be rather easily decreased by various procedures. The withdrawal of blood alone has been mentioned before. If spaced six days apart, we feel reassured (Table I, column 1) that this does not play a role in our studies. The injection of saline alone (Table I, column 2) can, however, occasionally decrease the total lipid concentration without affecting cholesterol values. Protein-free urine (column 3) decreased total lipid concentration markedly in six of eight experiments. In these six instances it would be difficult to distinguish the results from those reproduced in column 4 (Table I) where protein-containing nephrotic urine was used. It is thus probable that in the studies of Rosenman, Friedman, and Byers (7) similar results, possibly differing in intensity only in some of their experiments, would have been obtained if the nephrotic rats would have been infused with urine obtained from healthy control animals instead of having been subjected to a vena cava-ureteral anastomosis. It also is evident from column 5 (Table I) that dextran solution would have had the same effect on blood lipid and cholesterol concentration as that observed by the authors (7) when they infused nephrotic rats with albumin solution. We may assume that this

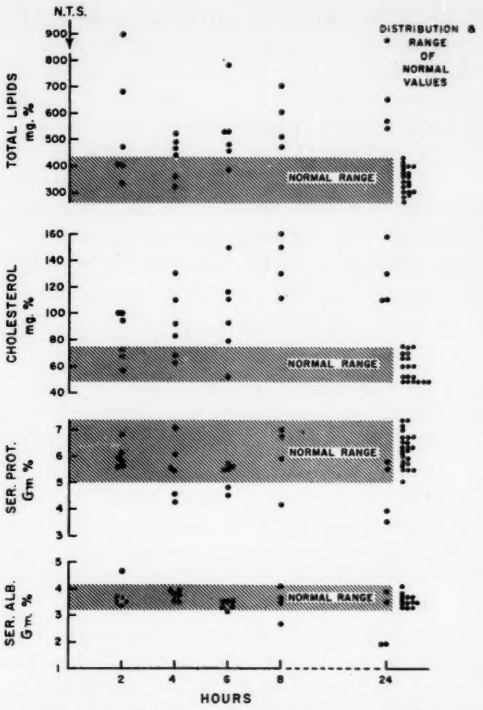

FIG. 2. TOTAL LIPID, CHOLESTEROL, SERUM PROTEIN AND SERUM ALBUMIN VALUES OBTAINED IN 26 RATS 2, 4, 6, 8 AND 24 HOURS, RESPECTIVELY, AFTER THE INTRAVENOUS INJECTION OF NEPHROTOXIC SERUM (NTS)

The shaded areas indicate the range of values obtained in 12 to 21 healthy rats of the same age and weight.

effect of dextran solutions is not due to hemodilution in that only nonedematous animals were used and hematocrit readings were unrelated to the observed lipid and cholesterol values obtained. The mechanisms of the hyperlipemia-reducing action of dextran is unclear. It has also been described by Cohen and Tudhope (14) in non-nephrotic human patients with hypercholesterolemia.

The coexistence of hypoalbuminemia and hyperlipemia is well-established in all forms of the nephrotic syndrome. We believe, however, that a causal relationship between them has not been demonstrated, in that: a) There is no relationship between severity of hypoalbuminemia and hyperlipemia; b) hyperlipemia does not succeed, but usually precedes the decrease in serum albumin concentration; and c) protein-free urine and dextran instead of albumin solutions decrease the

nephrotic hyperlipemia markedly. Clinical observation has furthermore taught us that hypoalbuminemia of comparable severity due to insufficient protein intake, as in kwashiorkor (15), or as noted in cases of hypocupremia and idiopathic hypoproteinemia (15), or as occasionally seen in chronic nephritis (16), is not necessarily associated with increased cholesterol or total lipid concentration. We therefore believe that a causal relationship between hypoalbuminemia and hyperlipemia has not been shown to exist as yet.

SUMMARY

1. No correlation between severity of hypoalbuminemia and hyperlipemia was noted in 19 nephrotic rats with renal disease of 1 to 11 months' duration.

2. The development of hypoalbuminemia and hyperlipemia was studied in 26 rats 2, 4, 6, 8 and 24 hours after the injection of anti-rat kidney sera. Hyperlipemia preceded, rather than succeeded, the hypoalbuminemia.

3. In nephrotic rats the intraperitoneal administration of nonprotein-containing rat urine decreased the hyperlipemia almost as regularly and markedly as when protein-containing nephrotic urines were used. The effect of dextran solutions on the experimental hyperlipemia was marked and regularly noted.

4. It is concluded that a causal relationship between hypoalbuminemia and hyperlipemia in the experimental renal disease of rats has as yet not been established.

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QUANTITATIVE DETERMINATION OF THE URINARY CORTISOL METABOLITES, "TETRAHYDRO F," "ALLO-TETRAHYDRO F" AND "TETRAHYDRO E": EFFECTS OF ADRENOCORTICOTROPIN AND COMPLEX TRAUMA IN THE HUMAN [1]

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Methods for the quantitative estimation of plasma cortisol concentration have been devised by Nelson and Samuels (1), Silber and Porter (2), and Peterson and Wyngaarden (3) who used the sulfuric acid-induced fluorescence developed by Sweat (4). Together with urinary procedures (5, 6) these techniques have been employed to determine the secretion of cortisol by the adrenal in normal and pathological conditions (7–9) and to evaluate the factors involved in the "removal" of cortisol from the plasma in surgery (10–13) and liver disease (14–16).

Although a considerable amount of work has been done in isolating and identifying such urinary metabolites of cortisol as THF [3] and THE (17–22) and their interconversions (23–25), much less has been done to relate the quantitative transformations of these urinary metabolites from cortisol under normal and pathological conditions. In 1953, deCourcy, Bush, Gray, and Lunnon (26) reported daily excretion values for two chromatographically separated urinary steroids corresponding in migration rates to THF and THE, in 10 normal men. The mean values were 212 μg. per 24 hours for THF and 1.5 mg. per 24 hours for THE, giving a ratio for THF:THE of 0.14. Romanoff, Seelye, Rodriquez, and Pincus (27) have recently published quantitative data on the excretion of THF and THE combined with allo-THF (28, 29) in one group of normal men and in a group of schizophrenic men. There was no essential difference in the ratio of THF:(THE plus allo-THF) between the two groups of men, and the ratio was reported as approximately 1:2. Cope and Hurlock (30) published data on the excretion of urinary THF and THE in several normal and surgical cases. Their results indicated increased excretion of these metabolites after surgery but did not show any other definite pattern of alteration of the metabolism of cortisol, although they state that "a tendency can be observed for tetrahydro-compound F excretion to rise more than does tetrahydrocortisone." In a preliminary report, Gold, Macfarlane, and Moore (31) showed that the relative proportions of the two urinary cortisol metabolites, THF and THE, were related to situations of "stress" and adrenocorticotropin (ACTH) administration. It is the purpose of this current report to supply additional evidence for alterations in the excretion of certain cortisol metabolites in the human in a variety of "stress" situations, such as surgery, bone trauma, burns and prolonged illness, and to compare the proportion of metabolites in these cases with normal individuals and with the urine of individuals receiving ACTH.

METHODS

Analytical methods

Urines were collected without preservative and were frozen until used.

[1] This work was suported in part by a grant from the Atomic Energy Commission. It was sponsored and supported in part by the Subcommittee on Metabolism in Trauma, Advisory Committee on Metabolism, Office of the Surgeon General, Department of the Army, through a contract (DA-49-MD-472) with Harvard University. The assistance of Winthrop Laboratories, Inc., and The Upjohn Co. is gratefully acknowledged.

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[3] Compounds referred to are the following: THF, tetrahydrocortisol (3α,11β,17α,21-tetrahydroxypregnan-20-one); allo-THF (3α,11β,17α,21-tetrahydroxyallopregnan-20-one); THE, tetrahydrocortisone (3α,17α,21-trihydroxypregnan-11,20-dione); F, cortisol (11β,17α,21-trihydroxypregnan-3,20-dione); E, cortisone (17α,21-dihydroxypregnan-3,11,20-trione).

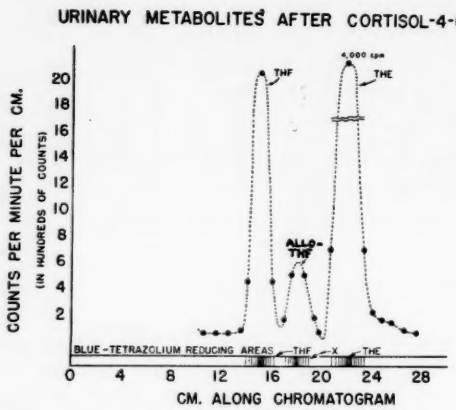

FIG. 1. THE DISTRIBUTION OF RADIOACTIVITY ON THE CHROMATOGRAM OF AN EXTRACT OF A URINE SAMPLE OBTAINED FROM A NORMAL INDIVIDUAL ADMINISTERED CORTISOL-4-C^{14}

The peaks of radioactivity correspond to the blue tetrazolium reducing steroid areas for both reference and urinary compounds. "X" is allo-THF.

Extraction procedure I. Thirty ml. of urine was adjusted to pH 5 with acetic acid and β-glucuronidase (Ketodase®) was added to a final concentration of 1,000 Fishman units per ml. The solution was incubated overnight at 47° C.

Extraction procedure II. Eight ml. or less of urine was treated as in Procedure I.

After incubation, the samples from either procedure were extracted with redistilled chloroform (3×20 ml.). The extracts were washed quickly with cold 0.5 N NaOH (2×5 ml.) and with distilled water (1×5 ml.). The combined aqueous washes were back-extracted with 15 ml. chloroform and the extract combined with the initial one. After drying with anhydrous Na_2SO_4 the chloroform solution was transferred to a 100 ml. round-bottom flask containing a 1 ml. well in its base. The chloroform was evaporated at 47° C. with the aid of a gentle stream of air and the sides of the flask were washed with chloroform to concentrate the residue in the well.

The residue obtained in Procedure I was made up to 0.5 ml. with ethanol and a suitable aliquot taken for paper chromatography. In Procedure II the entire residue was transferred to paper for chromatography.

Descending chromatography was a modification of one employed by Bush (32) and consisted of four volumes of toluene equilibrated with one volume of 75 per cent methanol in water. Chromatography was carried out at 32° C. using Whatman No. 1 filter paper. Although compounds THF and THE separated well in 6 hours, it was necessary to employ long "over-runs" of 16 to 39 hours to separate a third steroid, allo-THF, whose mobility was intermediate between THF and THE. The urinary allo-THF migrated with the same mobility as an authentic sample of the compound and after isolation of sufficient crystalline material from a urine pool, allo-THF was identified by its infrared spectrum.[4] This compound has recently been shown to be a normal component in human urine by Romanoff and co-workers (28) and Bush and Willoughby (29), and is apparently a metabolite of cortisol, as can be seen from Figure 1. Here the distribution of radioactivity was obtained by eluting, directly into planchets, 1 cm. strips from the chromatogram of an extract of a urine sample obtained from a normal individual administered 2 μc. of cortisol-4-C^{14} intravenously. Three peaks of radioactivity appear which correspond to the blue tetrazolium (BT[5]) reducing areas THF, allo-THF and THE.

Quantitative estimation of chromatographically separated steroids was achieved by spraying the chromatogram with a mixture of equal volumes of 0.2 per cent aqueous BT and 10 per cent sodium hydroxide. After remaining at room temperature for 10 minutes to assure full color development, the chromatogram was washed with tap water for 10 minutes and allowed to dry at room temperature. Blanks, standards and unknowns of equivalent areas were excised, cut into approximately one-fourth inch squares and shaken in closed vials for one hour with 1 to 3 ml. of the eluting agent, ethyl acetate: pyridine (1:4 by volume). Absorbances were read at 580 mμ in the Beckman spectrophotometer using microcuvettes.

Validation of analytical techniques

Mixtures of from 2 to 30 μg. each of THF, THE, F and E were chromatographed and estimated by the BT technique. The mean absorbances per μg. of steroid at 580 mμ plus or minus one standard error were, respectively, 0.036 ± 0.001; 0.038 ± 0.001; 0.035 ± 0.001; and 0.036 ± 0.004. Hence, there is adherence to the Lambert-Beer Law.

To demonstrate the recovery of steroid which has been subjected to chromatography, six samples of 21.4 μg. THE each were chromatographed along with reference standards to locate the THE areas. The former areas were eluted with ethanol and one-fifth volumes were assayed for steroid content by the Porter-Silber reaction (33). A nonchromatographed standard of 4.82 μg. THE was similarly estimated. The recovery ranged from 93 to 98 per cent, with a mean of 95 per cent.

The efficiencies of extraction Procedures I and II were determined. For Procedure I, six samples of 37 ml. of unhydrolyzed urine and six samples of 37 ml. of unhydrolyzed urine plus a mixture of 50 μg. each of THF, THE, F and E were extracted. Six controls contained the steroids only and were not extracted. For Procedure

[4] A sample of crystalline allo-THF was graciously contributed by Prof. T. Reichstein of the University of Basle. Dr. H. Rosenkrantz of the Worcester Foundation for Experimental Biology, Shrewsbury, Mass., kindly performed the infrared analysis.

[5] Obtained from Dajac Laboratories, Division of Monomer-Polymer, Leominster, Mass.

II, eight samples of 5 ml. of hydrolyzed urine and eight samples of 5 ml. of hydrolyzed urine plus a mixture of 20 μg. each of THF, THE and F were extracted. Four control samples contained the steroids only and were not extracted. All the samples were chromatographed and quantitatively evaluated with BT. For Procedure I, the per cent recoveries plus or minus one standard error were as follows: THF, 61 ± 2; THE, 87 ± 2; F, 88 ± 3; and E, 97 ± 2. Using Procedure II, the per cent recoveries were: THF, 86 ± 3; THE, 98 ± 2; and F, 100 ± 1. The more exhaustive extraction of Procedure II was employed in all later experiments.

Where indicated, quantitative values are corrected to 100 per cent recovery on the basis of recovery experiments. Quantitative standards of 20 μg. each of THF, THE and F were included on each sheet prepared for chromatography. Values for allo-THF in the BT estimation were calculated on the assumption that the ab-

TABLE I

The effect of surgical procedures on the relative proportions of urinary THF, allo-THF and THE

Patient	Age	Sex	Operation		Preoperation days				Postoperation days						
					3	2	1	Op.	1	2	3	4	5	6	7
A. G.	46	F	Abdominal hysterectomy	THF*		3.0		22.2	7.5	6.8	6.0	6.8	6.1	2.0	3.1
				THE		5.6		8.8	4.5	6.0	4.0	10.1	9.6	4.0	6.0
				Sum†		8.6		31.0	12.0	12.8	10.0	16.9	15.7	6.0	9.1
				Ratio‡		0.54		2.5	1.7	1.1	1.5	0.67	0.64	0.50	0.52
S. H.	45	F	Right ovariectomy	THF			2.3			3.9§	3.1	1.7	2.9	1.6	3.8
				allo-THF			1.0			0.4	0.4	0.4	0.5	0.5	1.1
				THE			2.6			1.0	1.4	1.2	1.4	1.9	3.2
				Sum			5.9			5.3	4.9	3.3	4.8	4.0	8.1
				Ratio			0.88			3.9	2.2	1.4	2.1	0.84	1.2
M. K.	46	F	Abdominal hysterectomy	THF*	1.8	1.7	1.7	2.4		2.8	2.2		2.0	2.3	2.1
				THE	2.3	2.2	2.1	1.6		2.4	2.1		3.4	2.9	3.6
				Sum	4.1	3.9	3.8	4.0		5.2	4.3		5.4	5.2	5.7
				Ratio	0.78	0.77	0.81	1.5		1.2	1.0		0.59	0.79	0.58
E. P.	43	F	Abdominal hysterectomy	THF*		2.1	1.5	15.1	8.8	8.2	4.8	2.4	3.5	2.3	1.7
				THE		3.4	2.3	6.3	5.5	5.1	4.1	2.4	3.6	2.0	1.9
				Sum		5.5	3.8	21.4	14.3	13.3	8.9	4.8	7.1	4.3	3.6
				Ratio		0.62	0.65	2.4	1.6	1.6	1.2	1.0	0.97	1.2	0.90
M. W.	44	F	Total hysterectomy	THF	0.96	1.04	0.92	5.1	2.9	1.9	1.8	1.5	1.3	0.92	1.7
				allo-THF	0.14	0.11	0.24	1.0	0.40	0.41	0.52	0.17	0.19	0.27	0.52
				THE	0.97	1.0	0.71	2.0	1.4	0.85	1.4	1.1	1.2	1.2	1.5
				Sum	2.1	2.1	1.9	8.1	4.7	3.2	3.7	2.8	2.7	2.4	3.7
				Ratio	0.99	1.0	1.3	2.6	2.1	2.2	1.3	1.4	1.1	0.77	1.1
M. H.	54	F	Bilateral ovariectomy	THF		3.0	3.3	25.0	3.9	4.6	2.5	2.0	1.5	3.1	3.3
				allo-THF		1.8	1.9	8.0	1.6	2.2	1.4	0.4	0.2	0.7	0.7
				THE		4.6	4.8	8.0	1.6	3.8	4.4	3.0	1.6	3.3	3.5
				Sum		9.4	10.0	41.0	7.1	10.6	8.3	5.4	3.3	7.1	7.5
				Ratio		0.65	0.69	3.1	2.4	1.2	0.57	0.67	0.94	0.94	0.94
P. L.	29	M	Repair knee ligament	THF				7.4	2.4						
				allo-THF				0.5	0.1						
				THE				2.5	1.8						
				Sum				10.4	4.3						
				Ratio				3.0	1.3						
J. L.	45	M	Sub-total gastrectomy	THF	2.7	2.6	3.4	14.6	7.6	5.2	5.4				3.3
				allo-THF	2.1	1.4	1.6	7.6	4.8	2.8	2.1				1.2
				THE	4.1	3.0	3.7	7.3	4.4	3.1	3.5				3.0
				Sum	8.9	7.0	8.7	29.5	16.8	11.1	11.0				7.5
				Ratio	0.66	0.87	0.92	2.0	1.7	1.7	1.5				1.1
L. L.	59	M	Fractured humerus set	THF				17.8	8.4	8.1	4.0	4.2	3.9		
				allo-THF				6.3	3.1	3.6	2.1	2.2	2.0		
				THE				8.1	4.2	6.4	4.7	5.6	5.9		
				Sum				32.2	15.7	18.1	10.8	12.0	11.8		
				Ratio				2.2	2.0	1.3	0.85	0.75	0.66		

* THF and allo-THF are combined.
† Sum is THF plus allo-THF plus THE. Values are corrected for extraction losses.
‡ Ratio is THF:THE. Other values are in mg. per 24 hrs.
§ Two day urine pool.

TABLE II

The effect of a surgical procedure on the relative proportions of urinary THF, allo-THF and THE—Detailed analysis *

Time	Operation	THF	Allo-THF	THE	Sum	THF	Allo-THF	THE	THF:THE
			μg./hr.				*% of sum*		
Day 1		114	89	172	375	30	24	46	0.65
Day 2	Difficult intubation;								
7 A.M.–3:30 P.M.	oper. postponed	502	217	378	1,115	47	19	33	1.4
3:30– 5:30 P.M.		152	118	152	422	36	28	36	1.0
5:30– 9:00 P.M.		300	154	258	712	42	22	36	1.2
9:00–10:45 P.M.		140	48	153	341	41	15	44	0.92
10:45– 4:00 A.M.		164	94	157	415	39	22	38	1.0
4:00– 7:00 A.M.		84	42	96	222	38	18	44	0.88
Day 3									
7:00–11:00 A.M.		144	72	158	374	38	19	42	0.90
11:00–12:00 P.M.	Subtotal	96	58	132	286	34	20	46	0.72
12:00– 1:00 P.M.	gastrectomy			No urine samples available					
1:00– 3:30 P.M.		247	122	271	640	39	19	42	0.91
3:30– 9:00 P.M.		720	400	336	1,456	49	27	23	2.1
9:00–11:00 P.M.		2,400	1,100	506	4,006	60	27	13	4.8
11:00– 3:00 A.M.		758	447	500	1,705	44	26	29	1.5
3:00– 7:00 A.M.		385	202	241	827	47	24	29	1.6
Day 4		315	156	180	651	48	24	28	1.7
Day 5		218	117	128	463	47	25	28	1.7
Day 6		226	89	146	461	49	19	32	1.5
Day 9		136	48	126	310	43	15	41	1.1
Day 15		93	40	124	257	36	16	48	0.75

* This case is J.L., aged 45, of Table I.

sorption per μg. of this compound was identical with that of the standards, THF and THE. Consequently, quantitative values for allo-THF are considered to be "relative."

Experimental subjects

Effects of surgical stress were studied in six women undergoing gynecological procedures and in three men undergoing gastrointestinal surgery, bone fracture repair, and knee ligament surgery. Urines were collected at 24 hour or shorter intervals during the study, pre-, intra- and postoperatively. Catheters were used where necessary to insure urine collection.

Effects of ACTH were determined in twelve men, eight of whom were normal volunteers. Of the remaining four, three were being treated for gonadal dysfunction and one

TABLE III

The effect of adrenocorticotropin (ACTH) on the relative proportions of urinary THF, allo-THF and THE

		No ACTH					ACTH (25 I.U. 8 Hrs. I.V.)				
		mg./24 hrs.					*mg./24 hrs.*				
Volunteer		THF	Allo-THF	THE	THF:THE	Allo-THF	THF	Allo-THF	THE	THF:THE	Allo-THF
	yrs.					%					%
W. A.	25	2.4	*	7.4	0.32	*	18.8	*	21.4	0.88	*
R. P.	19	3.1	*	6.5	0.48	*	15.6	*	19.0	0.82	*
W. S.	22	2.5	*	4.1	0.61	*	12.3	*	12.6	0.98	*
J. E.	22	2.1	2.1	2.5	0.84	31† (16–45)	8.8	7.6	6.1	1.4	34† (25–42)
W. F.	21	3.4	2.0	5.7	0.61	18† (6–36)	30.2	20.4	24.9	1.2	27† (14–41)
Z.	22	1.2	1.7	2.4	0.50	31† (21–43)	7.0	9.8	7.3	0.96	41† (26–50)
W. L.	21	2.7	1.3	3.6	0.75	17† (9–26)	17.2	6.5	10.8	1.6	19† (7–29)
A. V.	63‡	0.54	*	0.61	0.81	*	5.1	*	2.6	2.0	*
R. C.	66‡	3.6	*	5.5	0.65	*	18.5	*	13.5	1.4	*
F. W.	75§	0.69	*	0.39	1.7	*	5.0	*	1.8	2.8	*
P. G.	65‡	7.0	*	6.9	1.0	*	15.7	*	11.7	1.3	*
H. M.	50	2.2	0.76	2.0	1.1	15	7.6	2.7	4.9	1.6	18

* Allo-THF was not separated from THF.

† Urine samples were collected at four hour intervals. In these instances the mean value as well as the range are presented for the "allo-THF."

‡ Treated for gonadal dysfunction.

§ Cancer present.

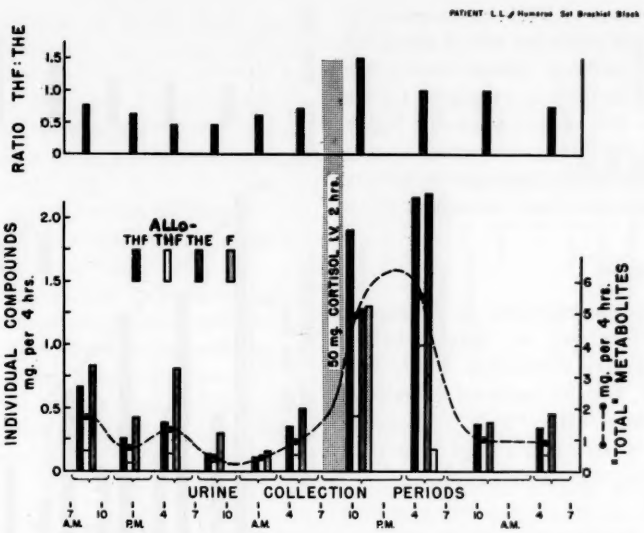

FIG. 2. THE EFFECT OF THE INTRAVENOUS ADMINISTRATION OF 50 MG. OF CORTISOL UPON THE URINARY CORTISOL METABOLITES OF PATIENT L.L.

Note the large proportion of cortisol that appears in the urine during the infusion period.

had cancer of the prostate. Urines were collected 24 hours prior to, during and 24 hours after ACTH administration. Twenty-five I.U. of ACTH was infused intravenously over eight hours.

Effects of cortisol were noted after the infusion of 50 mg. of cortisol over two hours to a man convalescing from a fractured humerus. Urine was collected periodically during the 24 hours preceding the infusion and during the 24 hour period which began with the infusion of cortisol.

Effects of severe burn trauma were observed in four patients. Three of the cases were fatal. Twenty-four hour urine collections were used. Catheters were used where necessary to insure urine collection.

Effects of prolonged stress were observed in a 50 year old man admitted with acute ulcerative colitis, who developed a pulmonary embolism, had a ligation of his inferior vena cava, developed an empyema and finally underwent a total colectomy. The effect of an ACTH infusion was observed both during the long period of illness and after successful convalescence. Urine samples were collected at 24 hour intervals.

RESULTS

Surgical "stress"

Table I expresses the daily excretion of the individual steroid metabolites, their sum, and the ratio THF: THE during the surgical procedures. In several instances where allo-THF was not separated from THF, the THF value includes allo-THF. It is apparent that the complex trauma of surgery is accompanied not only by elevations in the excretion of "total" urinary corticoids but also by increases in the relative proportions of THF: THE. In the instances in which allo-THF was evaluated no such clearly discernible pattern of variation with surgery was noted. There was, however, a consistent elevation in the rate of excretion of allo-THF during or shortly after the period of the "trauma."

A *detailed* analysis of the urinary corticoids from a patient undergoing surgery is presented in Table II. It illustrates the time relationship for the increase in the individual urinary metabolites both after the "difficult intubation" and after the gastrectomy. The increase in the proportion of THF: THE with the surgical procedures is clear, but a similar corelation is not observed between the relative per cent of allo-THF and these procedures.

Adrenocorticotropin

In all the cases presented in Table III the elevation of urinary cortisol metabolites after ACTH infusion was also accompanied by an increase in

the relative proportions of THF as compared to THE.[6] Although the mean per cent of allo-THF excreted was not markedly altered during the ACTH administration there is a tendency for the post-ACTH urines to contain a somewhat higher per cent of allo-THF. It is interesting to note the wide fluctuation in the per cent of urinary allo-THF for a given individual throughout a day.

Cortisol administration

The intravenous administration of 50 mg. of cortisol produced not only an increase in the amounts of the urinary metabolites THF, allo-THF, THE and F, but also increased the relative proportion of urinary THF. This is shown in Figure 2. It can be seen that a large proportion of the urinary steroid was cortisol.

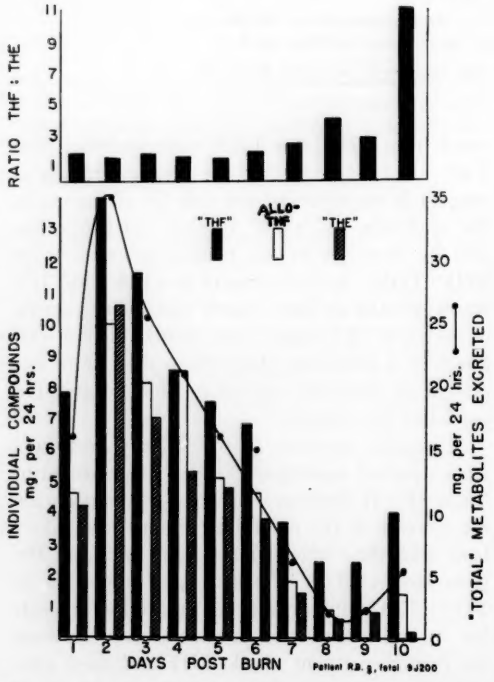

FIG. 3. URINARY CORTISOL METABOLITES IN A SEVERELY BURNED PATIENT, R.B., FEMALE, AGED 32 YEARS
The patient died on Day 11.

[6] A similar observation has been reported by Romani (34) in four subjects.

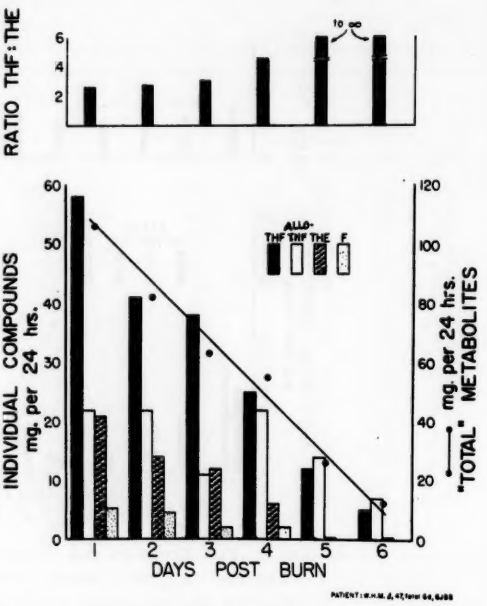

FIG. 4. URINARY CORTISOL METABOLITES IN A SEVERELY BURNED PATIENT, W.H.M., MALE, AGED 47 YEARS
The patient died on Day 7.

Severe burn trauma

The two fatal burns of Figures 3 and 4 indicate a high urinary titer of "total" metabolites at the period of initial trauma with a gradual decline to approximately normal values prior to death. All three metabolites, THF, allo-THF and THE, were excreted in greater than normal amounts. The huge excretion of "total" metabolites during Days one and two of Figure 4 suggests a very large secretion of cortisol by the adrenal.[7] This is consistent with the appearance of considerable amounts of cortisol "spilled over" into the urine on the first few days post burn. The ratio of THF : THE is elevated throughout and takes a sharp increase several days before death. The excretion of normal quantities of THF and allo-THF with markedly depressed excretions of THE suggests that the formation of THE from precursors is preferentially depressed.

In Figure 5 another fatal burn case is shown.

[7] Plasma 17-hydroxycorticoid values varied from 34 to 51 μg. F per 100 ml. plasma on the first two days post burn.

In this patient the ratio of THF: THE is considerably elevated through the seventh day post burn. By this day the excretion of THE has fallen to 1 mg. per 24 hours. On the eighth day infusions of cortisol were begun. There followed a rise in the amount of each urinary component *with the exception of THE.*

The last burn patient, shown in Figure 6, recovered. All three metabolites, THF, allo-THF (not shown in the figure) and THE, were elevated initially and gradually declined to normal values. The pattern of events was similar to that exhibited by the three fatal burns with the exceptions that the "total" metabolites excreted per 24 hours leveled off and the ratio of THF: THE returned to the normal value of 1.0 after having reached a maximum of 3.7.

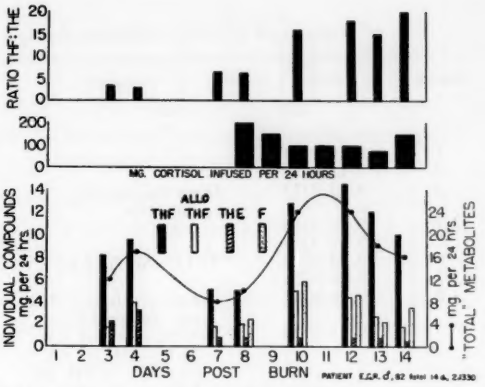

FIG. 5. URINARY CORTISOL METABOLITES IN A SEVERELY BURNED PATIENT, E.C.R., MALE, AGED 82 YEARS

The patient died on Day 15. Note that although large infusions of cortisol increased the excretion of THF, there is no increase in the quantity of THE excreted.

Prolonged stress

A quantitative study of the urinary metabolites from a patient severely ill for several months is shown in Table IV. It can be seen that: a) Allo-THF was not detectable on the chromatograms during the period of severe illness but was present in the convalescent period; b) the THF: THE ratio was elevated throughout the chronic stress period as compared to a "normal" day such as Sample 11; c) in Sample 6 during the period of surgical treatment the intravenous administration of 25 I.U. of ACTH produced a THF: THE ratio of 2.3, whereas in Sample 12, postconvalescence, the THF: THE ratio was only 1.4 after the same treatment while the "total" metabolites in each case were approximately the same; d)

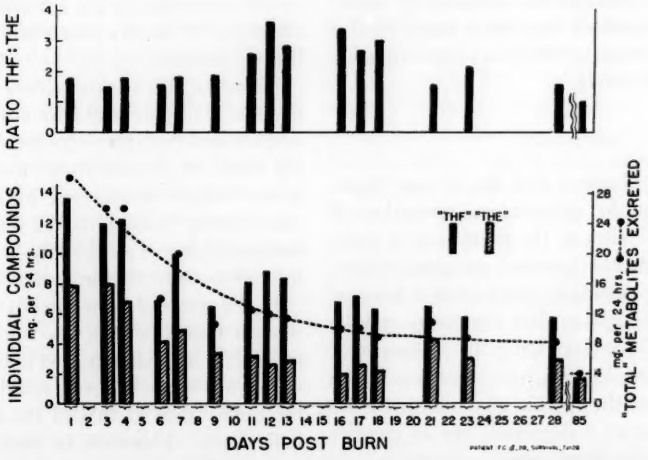

FIG. 6. URINARY CORTISOL METABOLITES IN A SEVERELY BURNED PATIENT, F.C., MALE, AGED 28 YEARS

The patient survived. Note the tendency for a further increase in the THF: THE ratio about two weeks post burn. Allo-THF, although present on the chromatogram, was omitted from the figure for purposes of clarity.

TABLE IV

Comparison of urinary metabolites during a prolonged illness with a postconvalescent period *

Sample	Date	Treatment	THF	Allo-THF†	THE	F	"Total"	THF:THE
			μg./24 hrs.				*mg./24 hrs.*	
	1956							
1.	9/10–9/11		3,660		1,890		5.6	1.9
2.	9/11–9/12		2,650		1,470		4.1	1.8
3.	9/12–9/13	Open drainage of empyema	3,670		2,260		5.9	1.6
4.	9/22–9/23		2,410		1,590		4.0	1.5
5.	9/23–9/24		3,010		2,580		5.6	1.2
6.	9/24–9/25	25 I.U. ACTH I.V.	11,600		5,100		16.7	2.3
7.	9/25–9/26		4,350		1,610		6.0	2.7
8.	9/26–9/27	Total colectomy; 200 mg. cortisol I.V.	38,300		5,600	7,180	51.1	6.8
9.	9/27–9/28	100 mg. cortisol I.V.; 25 I.U. ACTH I.V.; 40 I.U. ACTH gel I.M.	25,300		6,900	6,300	38.5	3.7
10.	10/15–10/16		3,450		1,650		5.1	2.1
Postconvalescence								
	1957							
11.	3/13–3/14		1,900	700	1,950		4.6	0.95
12.	3/14–3/15	25 I.U. ACTH I.V.	6,500	2,400	4,800		13.7	1.4
13.	3/15–3/16		2,550	1,100	2,300		6.0	1.1

* Patient H. M., male, 50 years, admitted 8/4/56 with acute ulcerative colitis, 8/8/56 developed pulmonary embolism, 8/9/56 ligation of inferior vena cava, 8/23/56 closed drainage of empyema.

† The absence of detectable quantities of allo-THF is not an artifact of the paper chromatography. Samples 8 and 12 analyzed individually and as mixed samples on the same chromatograms confirmed the "absence" of allo-THF.

during the period of prolonged stress the amount of "total" metabolites excreted in Samples 1, 2 and 4 were in the same range as in Samples 11 and 13, yet the THF : THE ratio was elevated in the stress period; e) although the excretion of "total" metabolites in Sample 8 was three times that of Sample 6 the excretion of THE was approximately the same in each sample.

DISCUSSION

It must be emphasized that the use of β-glucuronidase limits the quantitative estimation of the cortisol metabolites to the estimation of those metabolites which are excreted as glucuronides. Consequently the possibility exists that a lowered excretion of any single cortisol metabolite may be the result of: a) conjugation with a compound other than glucuronic acid, b) a decrease in the rate of filtration of the specific glucuronide by the kidney, as well as c) a depressed rate of conversion of cortisol to its metabolites.

It is clear that trauma (surgery, bone damage, burns) capable of eliciting an increase in output of urinary cortisol metabolites can also cause an increase in the per cent of urinary THF as compared to THE.[8] The greater increase in the amount of THF than THE after the administration of ACTH or cortisol suggests that this phenomenon may be directly related to the rate of cortisol secretion by the adrenal rather than to an alteration of factors concerned with the metabolism of cortisol.

However, the work of others (10–13) indicates that the ability to catabolize cortisol is reduced in surgery and that this reduction in activity involves the liver. It is conceivable then that the change in the relative quantities of THF and THE in the urine during trauma may be the result of *both* an increased adrenal secretion as well as an alteration in "metabolism" by the liver or other tissues.

In the series of burn cases it appears that there was an impairment in the conversion of cortisol to THE. Immediately post burn the THF : THE ratio was elevated above normal levels, but was in the same range as that of the surgically stressed individuals. However, in each of the four burn patients there was a further increase in this ratio after several days. In Figures 3 and 4 the largest

[8] Hemorrhage to the extent of 12 per cent of the total blood volume in 20 minutes did not elicit such a response in normal individuals.

rise in the THF: THE ratio occurred shortly before death, when the total metabolites had declined to approximately normal values. The severe depression in the relative amounts of THE excreted during the last two days prior to death of the patient of Figure 4 is strongly indicative of a preferential suppression of the reaction involving the oxidation of the hydroxyl group at position 11 to an 11-keto function. These findings tend to support the conclusions of Sandberg, Eik-Nes, Migeon, and Samuels (35) that there is an impaired metabolism of cortisol in dying patients.

That the oxidation of the 11-hydroxyl group may be inhibited is further supported by the data of Figure 5. It can be seen that by the seventh day post burn the quantity of THE excreted fell to 1 mg. per 24 hours. The infusion of large quantities of cortisol did not increase the amount of THE excreted although the amounts of THF and allo-THF rose markedly. This suggests that, although the mechanisms for the reduction and conjugation of ring A of cortisol are still active, some "physiological lesion" severely limits the conversion of cortisol to THE. The elevations of the THF: THE ratio on Day 10 of Figure 3 and the second and third weeks in the survival burn patient of Figure 6 may also represent a tendency toward the development of the same type of "physiological lesion."

An interesting illustration of altered cortisol metabolism is afforded by the patient of Table IV. This individual differed greatly from the "surgical" and "burn" patients in that his "stress," consisting of ulcerative colitis and pulmonary embolism with complications, was very prolonged, whereas the former types of stress were essentially acute. As shown in Table IV, there is an absence of detectable quantities of allo-THF during the period of extended stress and its appearance post convalescence is most striking. Although the possibility exists that allo-THF may not have been conjugated with glucuronic acid during the stress period and thus may not have been detected, such a possibility seems unlikely in view of the abundance of glucuronide conjugation occurring during the stress period.

Another indication of altered catabolism of cortisol in this same patient (Table IV) is the elevated ratio of THF: THE throughout the stress period as compared to the postconvalescence period, even though the "total" urinary metabolites in the stress period are approximately equal to those of the postconvalescent period.

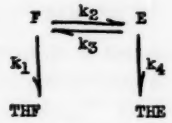

FIG. 7. SIMPLIFIED DIAGRAM OF ONE SEQUENCE IN THE CATABOLISM OF CORTISOL IN THE HUMAN (24)

Mechanisms for quantitative changes in urinary cortisol metabolites

A diagrammatic representation of several steps in the catabolism of cortisol, as shown in Figure 7, will help illustrate the experimental data. This scheme is based on the work of a number of investigators (17, 18, 36) and has recently been reviewed by Bush (24). The position of allo-THF in this scheme is not clear. It can be seen that a sudden increase in the secretion of F by the adrenal could lead to a transient increase in the relative quantity of THF formed if the resultant rates of Reactions k_2 and k_4 are slower than k_1 and k_3. In addition, it appears that either or both Reactions 2 and 4 are inhibited or that Reaction 3 is accelerated in complex trauma. Studies employing cortisol-4-C^{14} may help determine which of the kinetics of cortisol metabolism are altered.

It has been shown that there is a decreased rate of removal of cortisol from plasma in patients with cirrhosis (14, 16), in individuals undergoing surgery (10–12), and in dying patients (35). These effects have been attributed to altered liver function or to changes in hepatic flow. The role of the liver in producing the variations in urinary THF: THE ratios reported in this current investigation is not delineated.

The metabolites of cortisol which have been evaluated in this study represent only about 20 to 30 per cent of the total metabolites which appear in the urine (6, 16, 37). Another 30 per cent or more of urinary cortisol metabolites represented by the cortols and cortolones recently identified by Fukushima and co-workers (38) were not quantitatively evaluated in the current study. It may be that factors altering the kinetics of the conversion of cortisol to THF, allo-THF and THE may also involve the pathways concerned with the reduction of the C-20 keto group of cortisol.

SUMMARY

Several metabolites of cortisol were evaluated in the urine of subjects administered adrenocorticotropin and cortisol. Similar studies were made with patients undergoing such complex trauma as surgery or burns. The compounds evaluated were tetrahydrocortisol (3α,11β,17α,21-tetrahydroxypregnan-20-one), allo-tetrahydrocortisol (3α, 11β,17α,21-tetrahydroxyallopregnan-20-one), tetrahydrocortisone (3α,17α,21-trihydroxypregnan-11,20-dione), and cortisol (11β,17α,21-trihydroxypregnan-3,20-dione).

Observations showed an increase in both 1) the *rate of excretion* of each metabolite, tetrahydrocortisol, allo-tetrahydrocortisol, and tetrahydrocortisone, and 2) the *ratio of urinary tetrahydrocortisol to tetrahydrocortisone* in the following:

a) during and after *surgery,*
b) after the intravenous administration of 25 I.U. of *adrenocorticotropin* to normal subjects,
c) after the administration of 50 mg. of *cortisol,* intravenously, to one normal individual, and
d) in four patients with severe *burns.*

In three of the burn cases which were fatal the tetrahydrocortisol: tetrahydrocortisone ratio rose to extremely high values prior to death, suggesting the presence of factors which limit the conversion of cortisol to tetrahydrocortisone, *i.e.,* an inhibition of oxidation at the 11 position. There was no indication that the formation of allo-tetrahydrocortisol was grossly impaired. In one such patient the intravenous administration of large amounts of cortisol increased the daily excretion of tetrahydrocortisol and allo-tetrahydrocortisol but failed to increase the quantity of tetrahydrocortisone excreted.

The tetrahydrocortisol: tetrahydrocortisone ratio was elevated for an entire month in a chronically ill patient suffering from ulcerative colitis, pulmonary embolism, and empyema and having undergone major abdominal surgery. Allo-tetrahydrocortisol was not detected during this period. After five months' convalescence this patient showed a normal urinary pattern; allo-tetrahydrocortisol was present.

It is suggested that trauma in man can give rise to variations in the overall metabolism of cortisol.

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ADDENDUM

Gold and Moore demonstrated by *in vivo* isotope dilution procedures that THF and THE are not "interconvertible" in the normal subject (39). Details of these experiments are being prepared for publication.

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A METHOD FOR THE DETERMINATION OF FORMIMINOGLUTAMIC ACID IN URINE [1]

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Formimino-L-glutamic acid, an intermediate in the metabolism of histidine (1, 2), accumulates in the urine of folic acid-deficient rats (3–7). The urinary formiminoglutamic acid has also been reported to be increased in macrocytic anemia of pregnancy and in patients treated with folic acid antagonists (8–10). These findings can be attributed to a lack of tetrahydrofolic acid (THF), since enzymatic studies have shown (11–14) that THF is required for the further metabolism of formiminoglutamic acid.

The accumulation of formiminoglutamic acid in the urine can be used as a test for folic acid deficiency both in rats and in patients. The available assay for formiminoglutamic acid, based on the microbiological assay of the glutamic acid formed from formiminoglutamic acid by heating (4, 9), is relatively inconvenient and inaccurate. The assay is complicated by the fact that glutamic acid and glutamine are normally present in urine, as well as by the variable conversion of these compounds to pyrrolidone carboxylic acid during the heating step.

In this paper a sensitive spectrophotometric method is presented for the assay of formimino-L-glutamic acid in the urine. This method is based on enzymatic reactions (*1*) and (*2*) [Diagram 1] which we have recently (14) demonstrated to be involved in the metabolism of formiminoglutamic acid.

The amount of 5,10-methenyl-THF formed in reaction (*2*) is easily measured spectrophotometrically, since this compound has a high absorption in the ultraviolet with an extinction coefficient of 25,000 at 350 mμ (15–18). During the course of the reaction, however, 5,10-methenyl-THF is converted nonenzymatically to 10-formyl-THF in accordance with reaction (*3*).[2] The latter compound, however, is completely reconverted to 5,10-methenyl-THF upon acidification.

In the quantitative procedures presented here,[3] urine is incubated in phosphate buffer with a mixture of the transferase, cyclodeaminase and tetrahydrofolic acid. The mixture is then acidified, and the concentration of 5,10-methenyl-THF is calculated from the optical density at 350 mμ.

EXPERIMENTAL

Enzyme preparation. Commercial frozen hog liver is used as the starting material; this can be stored at −20° C. for as long as six months without loss of activity.

Step A. For the preparation of "acetone powders," 100 gram portions of hog liver are homogenized in a Waring blendor with approximately 500 ml. of acetone (technical grade) at room temperature for one minute; the mixture is then filtered rapidly through a large Büchner funnel. The moist filter cake is homogenized again with acetone, refiltered, passed through a coarse screen, and dried at room temperature overnight. The resultant powder can be stored over a drying agent *in vacuo* at −20° C. for as long as six months.

Step B. Extracts are prepared by gently stirring 160 grams of acetone powder with 1,600 ml. of water at room temperature. After 15 minutes the suspension is centrifuged in an angle centrifuge at 20,000 × G [4] for 15 min-

[1] Preliminary reports on this method have been presented at the meetings of the American Society of Biological Chemistry, April, 1957, and the American Society for Pharmacology and Experimental Therapeutics, September, 1957.

[2] The nonenzymatic conversion of 5,10-methenyl-THF to 10-formyl-THF at pH 7 is catalyzed by the phosphate buffer. This reaction is also rapidly catalyzed by the enzyme cyclohydrolase (14, 16, 17). This activity is not present, however, in the partially purified preparations described in this paper.

[3] Silverman, Gardiner, and Condit (19) have recently developed a method for formiminoglutamic acid estimation which depends on the formation of 10-formyl-THF from formiminoglutamic acid and folic acid in a crude chicken liver homogenate. The latter contains the enzymes mentioned above plus a folic acid reductase. In this precedure the 10-formyl-THF is converted to 5-formyl-THF by heating, and the latter is assayed microbiologically.

[4] Lower centrifugal speeds are satisfactory at this point.

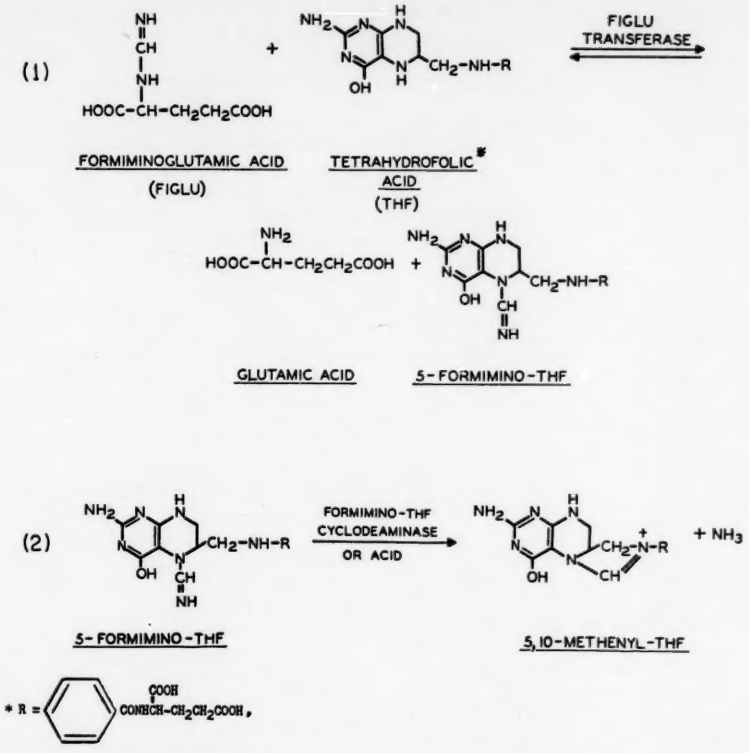

DIAGRAM 1

utes, and the supernatant solution collected.[5] Subsequent steps are carried out at 1 to 3° C.

Step C. Three hundred grams of ammonium sulfate is dissolved in 1,400 ml. of this supernatant solution. After approximately one hour the precipitate is collected by centrifugation at 20,000 × G for 15 minutes.[4] The precipitate is thoroughly suspended in 30 to 50 ml. of H_2O and stored overnight. The suspension is then centrifuged for two hours at 30,000 × G in an angle centrifuge, and the supernatant solution is discarded. The precipitate is then dissolved in 38 ml. of 0.2 M sodium acetate; after four hours the solution is centrifuged, and any insoluble material is discarded. This enzyme fraction is used for the assay procedure.

This preparation contains both the transferase and the cyclodeaminase. It has been stored with little loss of activity at − 20° C. for over six months, at 0° C. for over

[5] The enzyme purification procedure presented here has been very reproducible, and enzymatic assays have not ordinarily been carried out after each step. If desired, however, the following rapid assay can be used to follow the purification procedure. Although more precise assays for each enzyme activity are available (to be published), they are not necessary for the purposes of this paper.

The assay mixture is similar to that described below under "assay," and contains 0.1 ml. of 1 M potassium phosphate buffer (pH 7.2), 0.1 ml. of the tetrahydrofolic acid–mercaptoethanol solution, 5 micromoles of sodium formiminoglutamate, enzyme solution, and water to 1 ml. After five minutes at 25° C., 0.3 ml. of 10 per cent perchloric acid is added. The mixture is heated for one-half to one minute in a boiling water bath, cooled immediately in ice water, centrifuged, and the optical density of the supernatant solutions measured at 365 mμ against a suitable blank without formiminoglutamic acid. Under these conditions 0.01 ml. of this extract results in an increase in optical density of 0.6 to 1.0.

The same assay procedure can be used to follow Step C, using suitable dilutions of the enzyme. Step C results in a purification of approximately one hundredfold with about a 50 per cent yield of both the transferase and the cyclodeaminase.

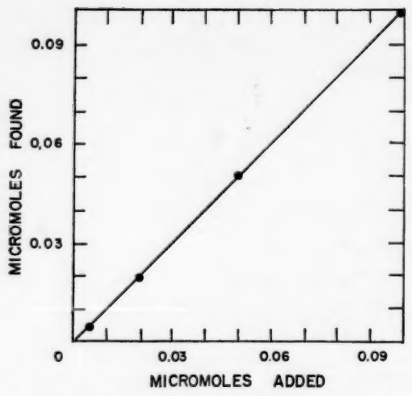

FIG. 1. RECOVERY OF FORMIMINOGLUTAMIC ACID ADDED TO URINE

Varying quantities of formiminoglutamic acid were added to 0.45 ml. aliquots of human urine. The mixture was then assayed for the formiminoglutamic acid content.

three months, and as a lyophilized powder at − 20° C. for over three months. Details on the further purification and separation of these enzymes will be published separately.

Tetrahydrofolic acid. Folic acid is reduced by the method of Jaenicke and Greenberg, as modified by Rabinowitz and Pricer (18). Solutions are prepared by dissolving 88 mg. of tetrahydrofolic acid in 18 ml. of 1.1 M mercaptoethanol plus 2 ml. of 0.2 N KOH. These solutions are usually 70 to 80 per cent pure, and contain 7 to 8 micromoles of *dl*-THF per ml. They are stored in test tubes *in vacuo* in the dark at 0° C., and have been stable for at least three months. Aliquots are transferred to small test tubes for daily use. The synthetic procedures used for the preparation of *formiminoglutamic acid, formiminoaspartic acid,* and *formylglutamic acid* have been described previously (2, 20).

Assay procedure. Urine (containing less than 0.1 micromole of formiminoglutamic acid), 0.25 ml. of the enzyme preparation, 0.1 ml. of tetrahydrofolic acid solution, 0.1 ml. of 1 M potassium phosphate buffer (pH 7.2), and water up to 1 ml. are incubated in narrow 3 ml. test tubes at 25° C. for 30 minutes. A "control" mixture is prepared in the same way except for the omission of enzyme. At the end of the incubation period 0.3 ml. of 10 per cent perchloric acid[6] is added; after two to two and one-fourth hours the solutions are centrifuged,[7] and the optical density at 350 or 365 mμ is read against the "control" cuvette. If the optical density is too high, the readings can be taken at 380 mμ where the extinction coefficient is lower. The optical density readings were corrected for a small enzyme blank (0.03 to 0.09). This value was obtained by incubating buffer, enzyme and THF; after the addition of perchloric acid (as above), the optical density was read against a similar mixture without enzyme.

The spectrophotometric readings are made in semimicrocuvettes (1.0 to 1.5 ml. volume) with a 1 cm. light path. The optical density obtained with 0.01 micromole of formiminoglutamic acid in 1.3 ml. volume under these conditions is 0.19 at 350 mμ, 0.17 at 365 mμ, or 0.11 at 380 mμ. Therefore, the quantity of formiminoglutamic acid in micromoles in the aliquot used is calculated by multiplying the corrected optical density by 0.052 at 350 mμ, 0.059 at 365 mμ, or 0.088 at 380 mμ. The sensitivity of the procedure is approximately 0.001 micromole.

Recovery of formiminoglutamic acid from urine. Good recovery is obtained when varying quantities of formiminoglutamic acid are added to water or to urine (Figure 1). Duplicate assays usually agree within 0.001 micromole, even when the determinations are carried out with different enzyme and tetrahydrofolic acid preparations. No inhibitory effects are observed with rat urine or with human urine, even after amethopterin (methotrexate) treatment (21).

Specificity. With the assay conditions used, no reaction (< 0.002 micromole) is obtained with 10 micromoles of L-methionine, L-serine, sodium formate, L-histidine, ammonium chloride, L-glutamic acid, formamide, formiminoglycine or glycine.

The enzyme preparation reacts very slowly with both formylglutamic acid (13, 22) and with formiminoaspartic acid. With 1 to 10 micromoles of either formylglutamic acid or formiminoaspartic acid, a reading equivalent to approximately 0.06 micromole of formiminoglutamic acid is obtained. There is no evidence at present that urine contains either formiminoaspartic acid or formylglutamic acid.

The enzyme preparation still has traces of urocanase.[8] However, even with 10 micromoles of urocanic acid, a maximum of 0.013 micromole of formiminoglutamic acid is formed. Thus, no significant error is introduced in the urinary assays at the levels of urocanic acid usually found in urine. Any error due to urocanic acid can be detected by the alkaline hydrolysis procedure described below, since urocanic acid is relatively stable under these condi-

[6] The perchloric acid is added both to deproteinize the incubation mixture and to convert formiminotetrahydrofolic acid and 10-formyltetrahydrofolic acid to 5,10-methenyl-THF.

[7] Alternatively, the mixture, after the addition of perchloric acid, is heated for one-half to one minute in a boiling water bath, cooled immediately in ice water, centrifuged, and the optical density of the supernatant solutions measured.

[8] The enzyme preparation contains approximately 0.3 per cent of the urocanase activity found in the crude extract. A preparation entirely free of urocanase can be obtained by treating 20 ml. of the enzyme solution with 2.0 ml. of a 3 M sodium acetate buffer [NaAC/HAc = 90/10] at 0° C. After four hours the precipitate is collected by centrifugation at 30,000 × G for 30 minutes, and dissolved in 20 ml. of 0.1 M triethanolamine sulfate buffer (pH 7.1). This preparation represents a one thousandfold purification of both the transferase and the cyclodeaminase.

tions (> 95 per cent remaining after autoclaving for two hours).

Although no additional procedures have been used routinely for the further identification of the formiminoglutamic acid in urine, several procedures are available for this purpose:

1. *Alkaline hydrolysis.* Formiminoglutamic acid is very unstable at an alkaline pH. It has a half-life at 25° C. of 10 minutes in 1 N KOH, 20 minutes at pH 11.5, 70 minutes at pH 10.8, and 40 hours at pH 9.2. To destroy the formiminoglutamic acid, therefore, the urine is adjusted to pH 11.5 with KOH and incubated at 25° C. for four hours. This alkali treatment causes a decrease in the assay of over 97 per cent; the small residual reading is due to formylglutamic acid which accumulates during the mild alkaline hydrolysis, and, as indicated above, reacts slowly in the enzyme assay. To obtain complete hydrolysis, the following conditions are used. Two ml. of urine is neutralized with KOH; 0.1 ml. of 6 N KOH is then added, and the mixture is autoclaved for two hours at 15 pounds pressure. We have observed complete hydrolysis under these conditions in all the urine samples that we have tested.

Formiminoglutamic acid is very stable in acid at 25° C., showing no destruction after several months in 0.1 N acetic acid, 0.1 N HCl, and 6 N HCl. At pH 7 it is relatively stable at 0° C., but is destroyed (> 98 per cent) by autoclaving for two hours (4).

2. *Alkaline ferricyanide-nitroprusside reaction.* Formiminoglutamic acid in urine may also be determined colorimetrically by a modification of the method used by Rabinowitz and Pricer for formiminoglycine determination (23, 24). Urine plus H_2O (0.5 ml. volume) is mixed with 2 ml. of saturated sodium borate and 0.5 ml. of the ferricyanide-nitroprusside reagent (4 grams each of NaOH, potassium ferricyanide, and sodium nitroprusside in 120 ml. of H_2O). The optical density at 485 mμ is read after 30 minutes at room temperature against a corresponding blank. The latter is prepared by preincubating 0.3 ml. urine with 0.1 ml. of 2.5 N KOH for 2 hours at 25° C. to destroy the formiminoglutamic acid present; the mixture is then neutralized with 0.1 ml. 2.5 N HCl, and assayed by the above procedure.

Under these conditions an optical density of 0.60 is obtained with 1 micromole of formiminoglutamic acid. Thus, this method is considerably less sensitive than the enzymatic procedure. The colorimetric assay has been satisfactory, however, for following the excretion of formiminoglutamic acid in the urines of folic acid-deficient rats, in which large amounts of this material are excreted.

3. *Chromatography.* Aliquots of urine containing large quantities of formiminoglutamic acid can be chromatographed on Whatman No. 1 paper in *t*-butanol 70, formic acid 15, H_2O 15 ($R_f = 0.52$) (2, 20, 25) or in the phenol-phosphate-citrate solvent of Broquist (9). After removal of the solvent, the paper is placed in an atmosphere of ammonia for two hours to hydrolyze the formiminoglutamic acid (20). The ammonia vapors are removed by aeration; the paper is sprayed with a ninhydrin solution, and the color is developed at room temperature. A control paper is usually run without the ammonia treatment, since formiminoglutamic acid gives only a very weak spot with ninhydrin under these conditions.

Formiminoglutamic acid may also be chromatographed on Dowex 50 and Dowex 1, as previously described (2, 4, 20).

DISCUSSION

Formiminoglutamic acid has been shown to accumulate in the urine in folic acid deficiency (3–10). A quantitative determination of urinary formiminoglutamic acid is useful for following the excretion of this compound in folic acid-deficient animals (3–7), in patients treated with anti-folic acid compounds (9, 10), and in certain macrocytic anemias (8).

The method presented here affords a simple, specific spectrophotometric procedure for the determination of formiminoglutamic acid, which is applicable to studies on its excretion in urine. We have recently used this method for following the excretion of formiminoglutamic acid in the urine during the development of a folic acid deficiency in rats; this was induced by feeding a folic acid-deficient diet, containing succinylsulfathiazole. These data, which will be reported in greater detail elsewhere (7), are summarized in Table I.

In the accompanying paper (21) the use of this procedure to assay the formiminoglutamic acid concentration in various human urines has been described.

SUMMARY

A sensitive spectrophotometric method is presented for the assay of formiminoglutamic acid in the urine.

TABLE I

Effect of folic acid deficiency on formiminoglutamic acid excretion

Day of diet*	Folic acid administered	Formiminoglutamic acid excreted
	0.05 μM/rat/day	*μM/7 hr. period*
7	0	0.22
14	0	1.29
21	0	2.06
28	0	9.66
35	0	21.2
41	0	36.5
43	+	0.38
45	+	0.18

* A folic acid-deficient diet, containing 1 per cent succinylsulfathiazole, was fed to six weanling rats. Further details on this experiment will be published subsequently in a more detailed study of the urinary changes in folic acid deficiency (7).

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URINARY EXCRETION OF FORMIMINOGLUTAMIC ACID BY HUMAN SUBJECTS AFTER ANTIFOLIC ACID THERAPY[1]

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In the accompanying paper a sensitive and specific enzymatic method is described for the measurement of formiminoglutamic acid (1). In this paper data are presented on the application of this method for the assay of formiminoglutamic acid in the urine of a variety of patients.

Formiminoglutamic acid is an intermediate in histidine metabolism, and requires tetrahydrofolic acid for its further metabolism. Previous results (2–5) have demonstrated that the urinary excretion of this compound is markedly increased in folic acid-deficient rats. Formiminoglutamic acid has also been reported (6, 7) in the urine of several patients receiving folic acid antagonists, although the techniques available for these studies were relatively cumbersome and inaccurate.

Using the enzymatic procedure for the assay of formiminoglutamic acid, we have found that small amounts of this compound can often be detected in the urine of adults and children. A marked elevation in formiminoglutamic acid excretion was noted after amethopterin (methotrexate) therapy in children with acute leukemia.

METHODS

Timed urine samples were obtained from 3 healthy adults and from 20 patients on the Medical and Pediatric Services of the Beth Israel Hospital. In addition, specimens were obtained from 22 children undergoing therapy for acute leukemia at the Children's Cancer Research Foundation. Sixteen of the latter group were receiving the antifolic acid drug, amethopterin, in the amounts listed in Table III. Pertinent clinical data are summarized in Tables I to III.

Urine samples were refrigerated immediately, and either analyzed for formiminoglutamic acid within 18 hours or frozen within that time and kept at $-10°$ C. until the analyses were performed. Urinary formiminoglutamic acid was measured by the procedure of Tabor and Wyngarden (1). Most urine samples containing large quantities of formiminoglutamic acid were subjected to the alkaline hydrolysis procedure described in the accompanying paper (1). In all instances there was a 95 to 100 per cent disappearance of the material reacting in the formiminoglutamic acid test.

RESULTS

Urinary excretion of formiminoglutamic acid in the nonleukemic children and adults varied from 0 to 0.51 micromole per hour (Table I).

The children with acute leukemia who had not received amethopterin for at least one week prior to the study excreted 0 to 1.18 micromoles of formiminoglutamic acid per hour (Table II). The three children in this group who had never been given the drug excreted 0 to 0.03 micromole per hour. Sixteen patients receiving amethopterin excreted quantities of formiminoglutamic acid in excess of 1.27 micromoles per hour; the excretion exceeded 8 micromoles in 11 of these cases (Table III). A rise in formiminoglutamic acid excretion was not apparent until after several weeks of amethopterin therapy, and often not until long after the patient had experienced a remission. Thus, Patient 33 excreted 0.59 micromole per hour 12 weeks following the institution of treatment, and 60 micromoles after 18 weeks; in Patients 26, 43 and 44 a similar phenomenon was observed. Following the cessation of therapy, Patients 34, 47 and 55 showed a sharp fall in formiminoglutamic acid excretion. The quantity of formiminoglutamic acid in the urine has no apparent relationship to the response of the leukemic process to therapy.

No evidence was found in any of the urines for any substances that would interfere with the formiminoglutamic acid assay. This was demonstrated by recovery experiments in which the urine samples were assayed after the addition of known increments of formiminoglutamic acid; 95 to 100 per cent recovery was obtained in all cases tested.

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TABLE I

Excretion of formiminoglutamic acid in nonleukemic subjects

Subject	Sex	Age	Clinical data	Urinary formiminoglutamic acid μM/hr.
Children				
2	M	12	Ulcerative colitis	0.18
5	M	10	Streptococcal pharyngitis	0.21
8	F	10	Repair of uretero-pelvic junction	0
45	M	11	Rhabdomyosarcoma	0.22
46	M	2	Neuroblastoma	0
89	M	15	Torsion of testicle	0
90	F	10	Fracture of fibula	0.39
Adults				
60	M	32	Normal control	0.40
61	M	35	Normal control	0
62	M	29	Normal control	0.50
82	M	65	Arteriosclerotic heart disease	0.19
83	M	65	Metastatic carcinoma	0.13
84	M	60	? Neurologic disease	0.51
85	M	59	Angina pectoris	0.25
86	M	67	Emphysema	0
87	M	19	Idiopathic thrombocytopenia	0.13
88	M	68	Pneumonia	0.31
91	F	72	Aortic stenosis, angina pectoris	0.03
92	F	68	Acute myocardial infarct	0.23
93	F	64	Acute myocardial infarct	0.35
94	F	64	Congestive heart failure	0.09
95	F	70	Angiocystic hamartoma	0.07
96	F	60	Acute myocardial infarct	0.08

DISCUSSION

The data presented above demonstrate the suitability of the assay procedure for the determination of urinary formiminoglutamic acid excretion. Preliminary data are presented on the excretion of this compound in a variety of cases, demonstrating that it is frequently detected in the urine of children and adults. The most striking results are represented by the increased excretion of formiminoglutamic acid in leukemic children during amethopterin treatment. The increased excretion in the amethopterin-treated cases is presumably due to the interference of the amethopterin with the formation of tetrahydrofolic acid (8), a compound required for the further metabolism of formiminoglutamic acid.

The increased excretion was often not seen until after several weeks or months of therapy. It should be noted that the quantity of amethopterin given was relatively small, so as to avoid toxicity. It is of considerable interest that in several patients a complete remission of the leukemia had taken place well in advance of an increase in formiminoglutamic acid excretion, or in formic acid excretion (9). If the increase in urinary formiminoglutamic acid and formic acid can be considered an index of folic acid deficiency (2–5), these observations may provide *in vivo* support for the thesis that leukemic cells have a greatly increased sensitivity to folic acid deprivation (10).

SUMMARY

An enzymatic procedure has been applied to the assay of formiminoglutamic acid in the urine of adults and children with a variety of disease states. Urinary formiminoglutamic acid was fre-

TABLE II

Formiminoglutamic acid excretion in children with acute leukemia not receiving amethopterin

Subject	Sex	Age	Clinical data	Previous amethopterin and interval since last given	Urinary formiminoglutamic acid μM/hr.
21	M	13	In remission on 50 mg. of 6-mercaptopurine daily	Yes; 5 months	0
23	M	4	In relapse; receiving adrenal steroids, chloramphenicol and novobiocin	Yes; 6 months	0
24	M	6	In relapse; receiving intravenous adrenocorticotrophic hormone, chloramphenicol, and novobiocin	Yes; 5 months	1.01
26	M	3	No previous therapy; receiving adrenal steroids	No	0
40	F	7	Amethopterin omitted 14 days previously because of severe toxicity (stomatitis); partial remission	Yes; 14 days	1.18
40	F	7	Amethopterin omitted 23 days previously	Yes; 23 days	0.42
50	M	1	No previous therapy	No	0.03
53	F	2	No previous therapy	No	0

TABLE III

Formiminoglutamic acid excretion in subjects with acute leukemia undergoing treatment with amethopterin

Patient	Sex	Age	Remission	Dosage of amethopterin	Duration of treatment	Urinary formimino-glutamic acid $\mu M/hr.$
26	M	3	Yes	1.25 mg. daily	4 weeks	0.06
26	M	3	Yes	1.25 mg. daily	10 weeks	8.8
27	F	5	Yes	1.25 mg. daily	3 months	1.3
28	M	4	Yes	2.5 mg. daily	6 weeks	11.0
28	M	4	Yes	2.5 mg. daily	18 weeks	11.2
29	F	4	Yes	1.25 mg. daily	11 months	2.8
30	M	4	No	1.25 mg. daily (and prednisone)	1 year	9.3
33	M	8	Yes	2.5 mg. daily (and prednisone)	3 weeks	0.17
33	M	8	Yes	2.5 mg. daily (and prednisone)	4 weeks	0
33	M	8	Yes	2.5 mg. daily (and prednisone)	10 weeks	0.68
33	M	8	Yes	2.5 mg. daily (and prednisone)	12 weeks	0.59
33	M	8	Yes	2.5 mg. daily (and prednisone)	15 weeks	5.0
33	M	8	Yes	2.5 mg. daily (and prednisone)	18 weeks	60.0
34	M	3	Partial	1.25 mg. daily	3 months	54.6*†
34	M	3		Amethopterin stopped 3 weeks previously		0.67
34	M	3		Amethopterin stopped 4 weeks previously		1.6
35	M	3	Yes	2.5 mg. daily	6 months	60.9
35	M	3	Yes	2.5 mg. daily	8 months	18.0
37	F	7	No	2.5 mg. daily (and prednisone)	2 months	2.4
41	F	8	Yes	2.5 mg. daily (and prednisone)	3 months	6.9
43	M	9	Yes	2.5 mg. daily (and prednisone)	3 weeks	0.18
43	M	9	Yes	2.5 mg. daily (and prednisone)	12 weeks	26.9
43	M	9	Yes	2.5 mg. daily (and prednisone)	16 weeks	42.9*
44	F	5	Yes	1.5 mg. daily	6 weeks	0.11
44	F	5	Yes	1.5 mg. daily	15 weeks	2.5
44	F	5	Yes	1.5 mg. daily	22 weeks	78.0
47	M	3	Early relapse	2.5 mg. daily	5 months	9.3
47	M	3	Early relapse	2.5 mg. daily	5½ months	62.5
47	M	3	Early relapse	Amethopterin stopped 2 weeks previously		5.6
47	M	3	Early relapse	Amethopterin stopped 3 weeks previously		0
55	M	3	In relapse	2.5 mg. daily	8 months	96.1
55	M	3	In relapse	Amethopterin stopped 3 weeks previously		0.62
56	M	6	Not yet complete (improving)	2.5 mg. daily	14 days	3.2
57	M	10	Yes	2.5 and 5.0 mg. on alternate days	1 month	9.9

* Further evidence for the excretion of formiminoglutamic acid by these cases was obtained by paper chromatography in *t*-butanol 70, formic acid 15, water 15, as described in the accompanying paper (1).

† During a 6 day period of hospitalization while this patient was receiving amethopterin therapy, 14 urine samples, excreted during known intervals, were collected. Formiminoglutamic acid excretion varied from 0.78 to 8.2 micromoles per ml., and from 24 to 130 micromoles per hour.

quently detected in small quantities, and was markedly increased in children treated with the folic acid antagonist, amethopterin.

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IRON ENZYMES IN IRON DEFICIENCY. II. CATALASE IN HUMAN ERYTHROCYTES

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In a previous paper we pointed out that while it was often assumed that the iron enzymes were "inviolate" in iron deficiency, this view was unsupported by any acceptable experimental evidence. Indeed, it was possible to demonstrate that in rats rendered iron deficient by a combination of bleeding and iron-poor diet, the cytochrome C content of the liver and kidneys was diminished markedly (1). As yet, these studies have not been extended to human subjects because of the difficulty in obtaining sufficiently large samples of cytochrome-containing tissues. However, another iron enzyme, the heme-protein complex catalase, is readily available for study in the red blood cells of patients, and it seems surprising that more attention has not been paid to this easily obtained and easily measured enzyme in the iron-deficiency state. A few measurements have been made of catalase activity in the organs of iron-deficient experimental animals and these have yielded contradictory results (2, 3). Studies in man have been limited in scope and have yielded difficult-to-interpret and inconclusive results. It is usually unclear whether the patients who were studied were iron-deficient or not (4–11). Furthermore, in nearly all instances the method of catalase estimation is clearly invalid; a catalase index was calculated by dividing the number of milligrams of peroxide destroyed in a fixed period of time by the red blood cell count. Since the destruction of H_2O_2 by catalase is a first order reaction, the quantity of H_2O_2 that has been decomposed at any given time is not a linear function of the amount of enzyme present. In addition, the incubation times used, up to two hours, were so long that virtually all the peroxide was destroyed, and the quantity of enzyme was no longer an important limiting factor in the reaction.

The study described in this paper was undertaken to clarify the effect of iron deficiency on catalase activity in the red blood cells of man.

MATERIALS AND METHODS

Patients. All subjects studied were patients or laboratory personnel of The University of Chicago Clinics. The normal group was composed of five males and four females whose bone marrow showed the presence of adequate iron stores or who were regularly taking iron orally as prophylaxis against recurrence of iron-deficiency anemia. The diagnosis of iron-deficiency anemia in the other 11 subjects was based upon the usual clinical criteria.

Blood samples. Blood samples were obtained by venipuncture. Heparin was used as the anticoagulant. Red blood cell counts, hemoglobin determinations, and hematocrits were carried out on fresh blood samples, using conventional techniques. Freezing and storing did not influence catalase activity, and in a few instances, the samples were stored in the frozen state before the catalase activity was determined.

Catalase activity determinations. Catalase activity was measured according to the method of von Euler (12). Five ml. of 1:2,000 and 1:4,000 dilutions of whole blood in distilled water was added to flasks containing 45 ml. of 0.0078 N H_2O_2 in 0.03 M phosphate buffer at pH 6.8. All reagents were held at ice water temperature. The amount of peroxide remaining at three, six and nine minutes was measured by titration of an aliquot with permanganate after the reaction had been stopped with sulfuric acid. At the dilutions used, the reaction rate was found to be proportional to the amount of blood present. von Euler has employed the unit "Kat F" to designate the catalase activity per Gm. dry weight of enzyme preparation in this system. Since we were interested in the catalase activity as related, not to dry weight, but to volume of red cells, the number of red cells, and the hemoglobin content of the red cells, we have arbitrarily defined Kat $F_{(v)}$, Kat $F_{(RBC)}$ and Kat $F_{(Hb)}$ as the catalase activity of 1 ml. of red cells, 10^{10} red cells, and 1 Gm. hemoglobin, respectively, acting in a 50 ml. volume. Plasma is devoid of catalase activity and does not potentiate or inhibit the enzymatic activity of red cells. It is therefore ignored in these calculations. Base 10 logarithms have been used in determining the reaction constant.

RESULTS

The catalase activity of red cells from normal and iron-deficient subjects is presented in Figures

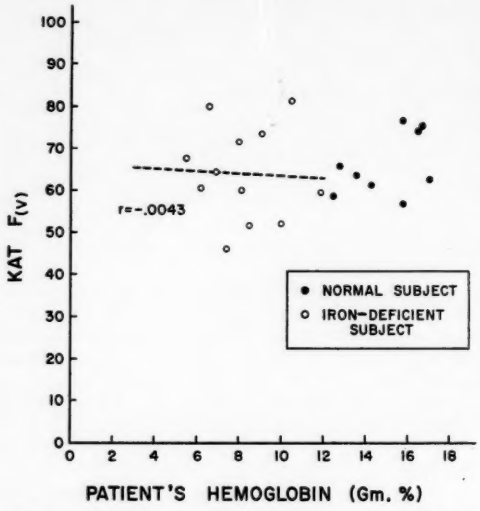

FIG. 1. THE RELATIONSHIP BETWEEN THE PATIENT'S HEMOGLOBIN LEVEL AND CATALASE ACTIVITY PER MILLILITER OF RED CELLS

1 through 3. It is apparent that the catalase activity per ml. of red cells was essentially the same in normal and iron-deficient subjects. This was true regardless of the severity of the anemia as indicated by the patient's hemoglobin level (correlation coefficient equals − 0.0043). The mean catalase activity of normal red cells was 65.9 ± 2.5 [1] Kat $F_{(V)}$ units while that of iron-deficient red cells was 64.0 ± 3.2 [1] Kat $F_{(V)}$ units. When expressed as catalase activity per 10^{10} red cells, however, the catalase activity of the red cells of the iron-deficient subjects was somewhat reduced. The degree of reduction of catalase activity was related to some extent to the severity of the iron deficiency as judged by the patient's hemoglobin level (correlation coefficient equals + 0.20). This is simply a reflection of the microcytosis of the red cells in iron deficiency. The mean catalase of normal red cells was 60.4 ± 2.1 [1] Kat $F_{(RBC)}$ units while that of iron-deficient cells was 54.8 ± 3.0 [1] Kat $F_{(RBC)}$

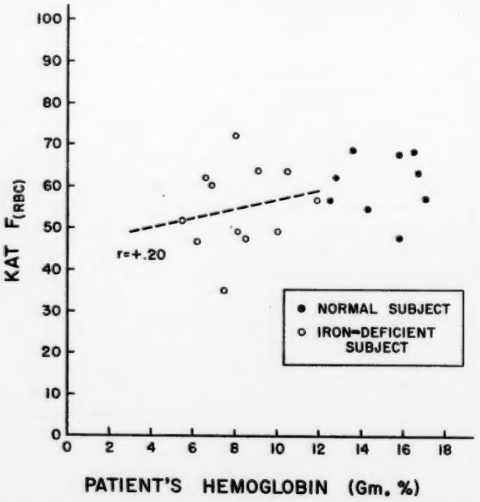

FIG. 2. THE RELATIONSHIP BETWEEN THE PATIENT'S HEMOGLOBIN LEVEL AND CATALASE ACTIVITY PER 10^{10} RBC

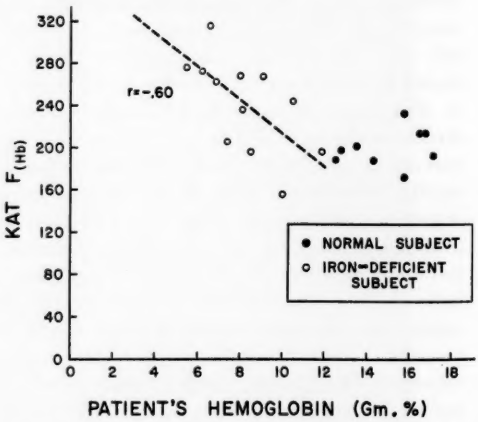

FIG. 3. THE RELATIONSHIP BETWEEN THE PATIENT'S HEMOGLOBIN LEVEL AND CATALASE ACTIVITY PER GM. OF HEMOGLOBIN

units. When the catalase activity was expressed as activity per Gm. of hemoglobin, a substantial increase in catalase activity in the iron-deficient as compared with the normal group was observed. The increase was inversely proportional to the hemoglobin level of the patient's blood (correlation coefficient equals − 0.60). This finding is a reflection of the reduction of hemoglobin concentration of the red cells which occurs in iron-deficiency anemia. The mean catalase activity of normal red cells was 198.2 ± 6.8 [1] Kat $F_{(Hb)}$ units while that of iron-deficient red cells was 241.4 ± 13.0 [1] Kat $F_{(Hb)}$ units.

No significant sex difference in red cell catalase

[1] One standard error.

activity was observed in the small number of samples that was studied.

DISCUSSION

It is apparent from these studies that even under conditions of severe iron need, the concentration of catalase in human red cells remains normal. If it is true, as has been suggested by Theorell, Beznak, Bonnichsen, Paul, and Akeson (13), that catalase and hemoglobin derive their heme groups from a common source, then it would appear that the catalase synthesizing mechanism has more success in competing for the limited amount of available heme than has the hemoglobin synthesizing mechanism. This would appear to be the reverse of the situation with cytochrome C, where it has been shown that the hemoglobin of iron-deficient animals returns to normal values while the cytochrome C content of liver and kidney is still very low (1).

SUMMARY

The catalase activity of the red cells of 9 normal subjects and 11 subjects with iron-deficiency anemia has been determined. Iron deficiency produced no change in the catalase activity per milliliter of red cells. Therefore, there was a slight decrease in catalase activity per 10^{10} red cells and a well marked increase in catalase activity per Gm. of red cell hemoglobin, reflecting the microcytosis and hypochromasia which occur in iron-deficiency anemia. These findings indicate that the mechanism synthesizing red cell catalase is more successful in competing for a limited quantity of iron than is the hemoglobin synthesizing mechanism, when only a small quantity of iron is available.

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THE RENAL EXCRETION OF DIGOXIN IN THE NORMAL YOUNG SUBJECT [1]

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Following the introduction of the embryonic duck heart assay method (1), and the radioactive isotope tracer technique (2), means were available for the quantitative study of the renal excretion of various cardioactive glycosides in both the experimental animal and man. It was but natural that the renal excretion of the digitalis glycoside, digitoxin, received the first attention of both the investigators employing the embryonic heart assay and those utilizing the radioactive method. Both groups agree exactly (3, 4) upon the amount of digitoxin excreted by man during the first 24 hours after administration. They also agree that the major portion of a given dose is destroyed and not excreted as intact digitoxin or a cardioactive metabolite of it. There is, however, some discrepancy in the quantitative estimates obtained by each group concerning the amount of cardioactive glycoside excreted after the first day of administration. This latter disagreement could be due to the fact that the group utilizing the embryonic duck heart method studied the renal excretion of glycoside in *young*, normal subjects, whereas the other group studied the same function in aged patients suffering from cardiac failure, subjects who have been found (3) to excrete far less unchanged digitoxin than the normal young subject.

Thus, if some information has been obtained about the renal excretion of digitoxin, no quantitative studies have been done concerning the renal excretion of other useful, frequently employed cardioactive glycosides. One of these is digoxin, whose excretion has been assumed to be far more rapid than that of digitoxin. This belief is based upon the comparative rapidity with which both the therapeutic and toxic effects of digitalization disappear after cessation of administration of the drug (5). However, no quantitative renal measurements have been made. It was decided, therefore, to measure by means of the embryonic duck heart method the renal excretion of this glycoside in normal young subjects. The results of this study published herein indicate that digoxin is indeed excreted by man far more rapidly than digitoxin.

[1] Aided by a grant from Burroughs Wellcome & Company, Inc.

METHODS

Eight normal young male and female subjects received 1.25 mg. of digoxin (Lanoxin®, Burroughs Wellcome) orally in a divided dosage of 0.25 mg. digoxin at five two-hour intervals. Twenty-four hour urine collections were begun immediately following the initial 0.25 mg. of the drug and continued over a period of four days. In addition, the urine was collected for 24 hours on the eighth day after initial ingestion in four of the eight subjects.

Digoxin was extracted from a 5 ml. aliquot of the 24 hour urine with 25 ml. chloroform by partition in glass stoppered test tubes. The phases were cleared by centrifugation; then the aqueous layer was aspirated off. Residual water and the interphase material were removed by filtration of the remaining tube contents through Whatman No. 5 paper into Erlenmeyer flasks (125 ml. with ground stoppers). The extraction tubes and filter paper were thoroughly washed with chloroform. After the extract was evaporated to apparent dryness, the last traces of chloroform fumes were driven off by the addition of alcohol and then the flasks brought to dryness. A standard volume of Tyrode's solution (5 ml.) was added to the flasks which were then shaken for one hour. The digoxin or cardioactive content of this extract was assayed by the embryonic duck heart method (1).

Recoveries of standard amounts of digoxin were made from samples of each subject's urine taken immediately before the initial ingestion of digoxin. The sensitivity of the method permitted the identification and measurement of amounts of digoxin of as little as 0.02 μg. per ml. of urine.

RESULTS

As shown in Table I, the maximal amount of digoxin is excreted during the first 24 hours after the ingestion of 1.25 mg., during which time an average of 169 μg. or 13.5 per cent of the total dose was excreted. A marked decline in the average excretion ensued in the subsequent two days, and on the fourth day three of the seven subjects

TABLE I

The renal excretion of 1.25 mg. digoxin

Subject	Age	Weight	Amount of digoxin in daily urine				
			Day 1	Day 2	Day 3	Day 4	Day 8
		lbs.			*μg.*		
T. I.	32	180	168	120	52		
C. Y. O.	34	135	207	172	88	85	N.D.*
W. H.	32	128	196	187	54	32	N.D.
J. P.	29	180	62	62	65	N.D.*	N.D.
M. S.	32	208	152	152	46	N.D.	
V. M.†	22	108	78	38	28	N.D.	
L. D.†	33	128	270	152	136	81	
E. D.†	32	118	224	150	63	45	N.D.
Average	31	148	169	129	66	61	
S. E. Mean			±23	±17	±11		

* N.D., no digoxin detectable (*i.e.*, less than 25 μg. of digoxin in 24 hour urine volume).
† Female.

examined failed to excrete detectable amounts of digoxin (*i.e.*, less than 25 μg.). None of four subjects tested excreted detectable amounts of the drug on the eighth day after its ingestion.

DISCUSSION

When these results are compared with those obtained on both normal and cardiac subjects given digitoxin (3, 6) it is clear that the human subject excretes a considerably larger fraction of ingested digoxin than of digitoxin, at least during the first 72 hours after administration. Whereas the young subject cannot excrete more than 5 per cent of a given dose of digitoxin in 24 hours or more than 14 per cent in 72 hours, he can excrete approximately 14 and 29 per cent, respectively, of administered digoxin during the same time intervals.

Although we were not able to detect digoxin in the urine of any subject eight days after the drug's ingestion, digitoxin could still be detected in the urine of the majority of subjects 15 days after its administration (3). However, this does not necessarily indicate that the former subjects were not still excreting some digoxin because our method of detection was considerably less sensitive for the presence of digoxin than digitoxin in urine. Thus our subjects could still have been excreting digoxin after the eighth day in daily quantities of less than 25 μg.—our critical endpoint of detection. Nevertheless it seems quite doubtful that the entire amount of digoxin was eliminated via the kidney. Rather it appears that, like digitoxin (3, 4), not only does the human subject eliminate digoxin by excretion of the intact substance and its cardioactive fragment but also probably metabolizes a large fraction of it.

SUMMARY

The renal excretion of digoxin was studied in eight normal young subjects by means of the embryonic duck heart technique. The excretion of this glycoside was found to be approximately twice as rapid as that of digitoxin during the first 72 hours after its administration.

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THE EFFECTS OF A VARIETY OF HEMODYNAMIC CHANGES ON THE RAPID AND SLOW COMPONENTS OF THE CIRCULATION IN THE HUMAN FOREARM [1]

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When a tracer substance which does not diffuse through the capillary walls is introduced into the brachial artery and the effluent blood is sampled continuously thereafter, a time-concentration curve of the labeled substance may be plotted which describes the velocity-flow pattern of the blood in the forearm (1). It was found that the descending limb of the time-concentration curve usually was biphasic. Evidence was presented which indicated that these biphasic curves were not due to differences in path lengths or to velocity flow through different tissues such as skin and muscle. It was suggested that this biphasic pattern was due to two basically different rates of flow in the highly anastomotic small vessel circulation (1–3). Since pressures are very nearly equal at either end of an anastomotic intercapillary or intervenular channel, flow may be sluggish in that channel. Direct observation of the circulation in the hamster cheek pouch disclosed that varying rates and direction of flow occur in the anastomotic channels—at times rapid, at times slow, at times surging, stopping or reversing.

It seemed of interest to determine what effect changes in both the local and general circulation would have upon the characteristics of this biphasic velocity-flow pattern in the forearm.

METHODS

The dye T-1824 was used as the tracer substance. The source of subjects, experimental technique, methods of analysis and calculation of results were identical to those previously described (1). The ratio of blood flow in the rapid component (f_1) to that in the slow (f_2) was estimated from the mean circulation times of the total curve (T), the rapid component (t_1), and the slow component (t_2) as described in a previous paper (1). The parameters f_1/f_2 and t_1/t_2 were used to estimate the ratio of the blood volumes (v_1/v_2) in the rapid and slow portions. The ratio of flows rather than absolute blood flows were estimated because the distribution of dye in the various branches of the brachial artery may not be uniform, and hence, the peripheral vasculature of the forearm cannot be regarded as a uniform mixing chamber (1).

Hyperemia restricted to the forearm under study was produced by the following techniques: 1) by the local intra-arterial injection of 5 mg. tolazoline (Priscoline®). After flushing of the skin had become plainly evident (usually after one to three minutes) the dye T-1824 was injected into the brachial artery; 2) by local exercise in which the subject squeezed a hand ergometer repeatedly at one-second intervals as forcibly as he was able until he could carry on no longer because of fatigue and aching of the forearm muscles. This varied from 65 to 124 squeezes in the 3 subjects. At this point the dye was injected through the previously placed intra-arterial needle without delay; 3) by reactive hyperemia in which the cuff of a blood pressure apparatus was wrapped on the upper arm and inflated to pressures 100 mm. Hg above systolic for a period of 10 minutes. Dye was injected immediately following release of the cuff.

In all of the hyperemia experiments it was necessary to double the dose of T-1824 in the experimental as compared to the control period. The reason for this was that the greatly increased volume flow reduced the dye concentrations. In all of the other procedures the dosage of the dye was the same in both the experimental and control periods.

Local injection of epinephrine and norepinephrine was carried out as follows: Epinephrine was diluted to a concentration of 0.4 μg. per ml. and levarterenol (norepinephrine) to 2.0 μg. per ml. in isotonic saline. These dilute solutions were injected intra-arterially through a three-way stopcock at a rate of 2 ml. per minute. When pallor of the skin occurred (usually after one to two minutes) an assistant attached the syringe containing the dye to the free arm of the three-way stopcock and injected it without delay. Following this the stopcock was immediately turned and the infusion of the pressor amine continued at the same even rate until the end of the collection period. The pressor amine also was added to the dye in a concentration similar to that present in the infusion. The blood pressure was recorded in the opposite arm. In no instance was a significant change in systemic arterial pressure observed.

When epinephrine and norepinephrine were given sys-

[1] Supported in part by United States Public Health Service Grants H-720 and H-1903 (National Heart Institute).

temically the infusion contained either 1 mg. of epinephrine or 2 mg. of norepinephrine per liter of isotonic saline. The infusion was connected via a three-way stopcock to a needle in a vein of the arm opposite to the forearm under study. A flask containing 1 liter of isotonic saline without added pressor amine was connected to the free limb of the three-way stopcock and infused slowly during the control period. Following the control experiment the stopcock was turned and the infusion of pressor amine was adjusted until a significant change in blood pressure and usually of the heart rate was obtained. The dye was then injected into the brachial artery of the forearm under study and the infusion rate of the pressor drug maintained at a level which produced a significant blood pressure change throughout the sample collection period. In the case of epinephrine the effective infusion rates averaged 15 ± 5 μg. per minute; for norepinephrine they averaged 36 ± 8 μg. per minute.

In the venous congestion experiments a narrow blood pressure cuff was wrapped proximally on the arm under study and covered with gauze bandage to prevent ballooning. The cuff was inflated to pressures of 40 to 60 mm. Hg for a period of five minutes before as well as throughout the injection and collection periods.

RESULTS

Control determinations were carried out immediately prior to the experimental period in 25 of the 47 subjects. Controls, being technically laborious, were not obtained in the remaining cases. Therefore, in analyzing the data, some with controls, some without, the coefficients of variation were used to obtain a combined estimate of the standard deviation. This seemed valid since the ratio of the standard deviation to the mean was nearly constant for the various groups (Table I). Using this combined estimate the t test for significance then was applied in the usual way. In the group of subjects given norepinephrine sufficient controls were carried out among these subjects to permit an intragroup comparison. The latter method of analysis was carried out in this case since the combined estimate provided suggestive but not conclusive indications of a significant difference.

Hyperemia

This was produced in several ways as described above: by local exercise (three cases), intra-arterial tolazoline (eight cases), and reactive hyperemia (four cases). Overall results indicated a highly significant shortening of T and t_2 ($p > 0.01$) with an increase in the ratios f_1/f_2 ($p > 0.01$) and v_1/v_2 ($p > 0.05$), indicating a marked relative shift of blood flow and volume to the rapid component (Tables I and II). These changes were most marked in the cases given intra-arterial tolazoline (Table I and Figure 1).

Control determinations were carried out in three cases with local exercise, in two of the subjects given intra-arterial tolazoline, and in one case subjected to reactive hyperemia. In each of these there were decreases in mean circulation times and increases in f_1/f_2 and v_1/v_2 as compared to the controls (Table I and Figure 1).

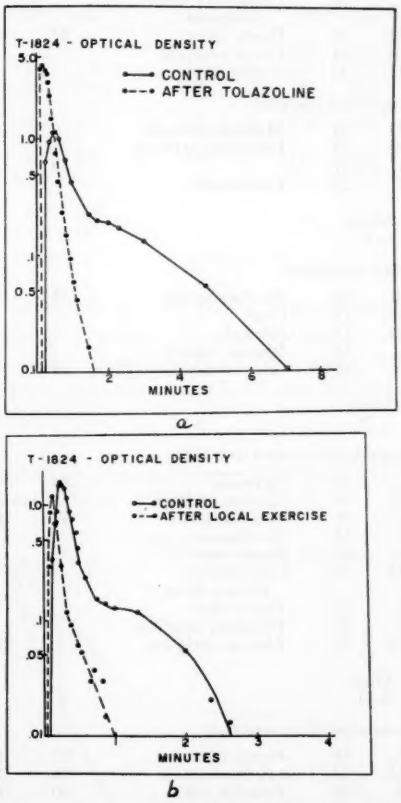

FIG. 1. TYPICAL EFFECTS OF HYPEREMIA ON THE BIPHASIC TIME-CONCENTRATION CURVES IN THE HUMAN FOREARM

Figure 1a (above) illustrates the effects of intra-arterial tolazoline. Figure 1b (below) shows the effects of local exercise.

TABLE I

Mean circulation times and ratios of blood flow and vascular volume in the rapid and slow components under various experimental conditions

Subject	Age	Diagnosis	Control					Experimental				
			T	t_1	t_2	f_1/f_2	v_1/v_2	T	t_1	t_2	f_1/f_2	v_1/v_2
	yrs.		*seconds*					*seconds*				
Hyperemia												
Local exercise												
J. H.	33	Peptic ulcer	102	49	247	2.7	0.5	64	51	222	12.0	2.8
W. C.	32	Urticaria	32	17	86	2.3	0.5	11	8	30	7.0	1.9
C. E.	24	Urethritis	58	19	96	1.0	0.2	21	18	61	11.0	3.6
Intra-arterial tolazoline												
W. A.	29	Urinary tract infect.	92	33	179	1.5	0.3	16	16	46	150.4	51.1
C. H.	46	Lung abscess						24	24	75	127.1	39.9
C. T.	26	Psychoneurosis						18				
W. H.	43	Acute infect. arthritis						17	15	46	16.2	5.3
L. P.	38	Pulmonary histoplasmosis						22				
A. M.	38	Peptic ulcer	82	15	97	0.2	0.1	16	14	60	33.3	7.9
M. R.	24	Boeck's sarcoid						19	15	42	5.2	1.9
D. C.	22	Peptic ulcer						22	12	65	4.3	0.8
Reactive hyperemia												
H. D.	35	Multiple sclerosis						23				
J. M.	32	Infectious arthritis						20	16	53	8.9	2.7
J. B.	37		49	24	79	1.2	0.3	27	13	46	1.4	0.4
R. J.	29	Pneumonia						17	9	49	3.9	0.7
Mean								25	18	68	33.8	10.7
S. D.								±14	±12	±60	±58.4	±19.4
Venous congestion												
B. B.	26	Psychoneurosis	38	30	113	8.6	2.2	149	55	223	0.8	0.2
A. M.	57	Gout						307	127	652	2.1	0.4
W. H.	35	Normal						210	54	251	0.3	0.1
J. G.	28	Rheum. spond.						209	125	406	2.3	0.1
R. H.	47	Gastroenteritis	89	31	123	0.6	0.2	215	48	320	0.6	0.1
Mean								218	82	370	1.2	0.2
S. D.								±57	±40	±172	±0.9	±0.2
Norepinephrine (intra-arterial)												
J. C.	35	Cirrhosis	83	43	135	1.3	0.4	93	46	129	0.6	0.2
D. H.	24	Rheum. arthritis	71	21	112	0.9	0.2	76	21	132	1.0	0.2
R. N.	46	Diab. mellitus	62	19	127	1.5	0.2	130	41	203	0.8	0.2
E. G.	48	No disease						61	26	122	1.7	0.4
L. G.	37	Peptic ulcer						120	36	231	1.3	0.2
C. M.	34	Convalescent rheum. fever						87	48	190	2.6	0.6
D. S.	33	Peptic ulcer						57	22	118	1.7	0.3
G. S.	42	Penicillin reaction						100	40	148	0.8	0.2
W. K.	35	Rheum. arthritis						127	24	73	1.1	0.2
Mean								95	34	150	1.3	0.3
S. D.								±26	±10	±45	±0.6	±0.14
Epinephrine (intra-arterial)												
P. N.	44	Peptic ulcer	92	16	168	1.0	0.1	63	18	139	1.7	0.2
C. M.	42	Psychoneurosis	44	14	86	1.4	0.2	41	19	97	2.6	0.5
G. S.	35	Irritable colon	40	15	75	1.4	0.3	55	21	106	1.5	0.3
M. M.	28	Peptic ulcer						54				
W. L.	23	Convalescent meningitis						34	13	78	2.1	0.3
F. H.	41	Peptic ulcer						61	38	104	1.9	0.7
E. D.	41	Fatty liver	75	20	125	0.9	0.1	60	16	126	1.5	0.2
Mean								52	21	108	1.8	0.4
S. D.								±10	±9	±22	±0.5	±0.4

TABLE I—*Continued*

Subject	Age	Diagnosis	Control T	Control t_1	Control t_2	Control f_1/f_2	Control v_1/v_2	Experimental T	Experimental t_1	Experimental t_2	Experimental f_1/f_2	Experimental v_1/v_2
	yrs.		*seconds*					*seconds*				
Epinephrine (systemic)												
S. W.	21	Convalescent pneumonia	47	18	117	2.4	0.4	34	12	81	2.2	0.3
M. K.	28	Psychoneurosis	72	27	180	0.7	0.2	103	40	144	0.7	0.2
D. C.	39	Peptic ulcer	57	16	80	0.6	0.1	47	15	80	1.1	0.2
H. S.	31	Pyeloneph. right	109	46	159	0.8	0.2	135	56	250	1.6	0.3
Mean								80	31	139	1.4	0.25
S. D.								±43	±21	±80	±0.6	±0.06
Norepinephrine (systemic)												
W. J.	38	No disease	22	19	67	3.6	3.2	25	16	86	7.0	1.3
O. Y.	33	Convalescent spontaneous pneumothorax	75	39	164	2.4	0.6	41	36	120	14.6	4.3
J. P.	30	Convalescent pneumonia	44	24	77	1.7	0.5	33	29	87	11.8	3.9
C. P.	26	Peptic ulcer						75	30	162	2.0	0.4
G. T.	34	Boeck's sarcoid	73	28	123	1.1	0.3	33	26	103	9.4	2.3
G. W.		Traumatic wrist drop	137	25	174	0.3	0.06	128	29	188	0.6	0.09
G. R.	26	No disease	97	18	132	0.4	0.1	70	20	128	1.2	0.2
Mean			75	26	123	1.6	0.8	58	27	125	6.7	1.8
S. D.			±38	±8	±44	±1.3	±1.2	±24	±7	±12	±5.5	±1.8
Mean (all controls)			65.7	25	125.2	1.6	0.5					
S. E. (all controls)			±22.7	±10.8	±43.8	±1.8	±0.7					

Venous congestion

In the five subjects studied there were marked prolongations of mean circulation times (Tables I and II, Figure 2). These were significant for T, t_1 and t_2 ($p > 0.05$, 0.05 and 0.01, respectively). Because of the nearly proportionate increase in all of these parameters the changes in f_1/f_2 and v_1/v_2 were not significant. It should be noted that the number of cases studied was not sufficient to detect small changes in these ratios. It would appear, however, that the predominant effects of congestion were to greatly retard blood flow and velocity in both components without much change in the relative flows and volumes.

Local epinephrine and norepinephrine

The changes observed after the intra-arterial infusion of epinephrine and norepinephrine were not significant. Following norepinephrine the average values in nine cases disclosed slight and insignificant increases in mean circulation times and reductions in f_1/f_2 and v_1/v_2 (Tables I and II). In the three cases in which control determinations immediately preceded the experimental period the only change consistent in all three was a slight to moderate increase in T (7, 15 and 109 per cent, respectively).

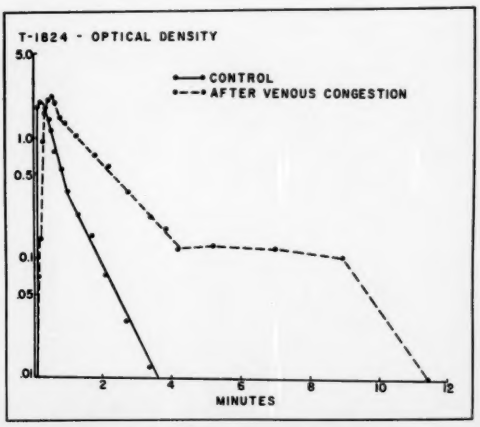

FIG. 2. EFFECTS OF VENOUS CONGESTION
See text for details.

TABLE II

Means, standard deviation of means, and statistical analysis of the

Procedure	No. of cases	T					t_1				
				Difference between means*					Difference between means		
		Mean	S.D.	Difference	S.D. of diff.	t	Mean	S.D.	Difference	S.D. of diff.	t
Control	25	66	23				25	11			
Hyperemia	15	25	14	41	6	6.5	18	12	7	3	2.2
Congestion	5	218	57	152	37	4.1	82	40	57	19	3.0
Epinephrine, local	7	52	10	14	11	1.3	21	9	4	5	0.8
Norepinephrine, local	9	95	26	29	17	1.7	34	10	9	6	1.5
Epinephrine, systemic	4	80	43	14	12	1.2	31	21	6	8	0.7
Norepinephrine, systemic	7	58	14	8	10	0.8	27	7	2	5	0.4

* Coefficients of variation used to obtain a combined estimate of the standard deviation. See text.

In the seven cases receiving intra-arterial epinephrine no significant changes were observed in the average values of T, t_1, t_2, f_1/f_2 and v_1/v_2 as compared to the combined series of 25 controls. In four of these subjects control determinations preceded the experimental period. Results in these cases failed to reveal consistent changes in the mean circulation times. In three of the four cases moderate increases in f_1/f_2 and v_1/v_2 occurred. This must be regarded as being of questionable significance since the average values for the entire group of seven failed to confirm such a trend.

The changes after norepinephrine and epinephrine locally, therefore, were not striking. After norepinephrine there seemed to be a moderate delay in mean circulation time but this was not statistically significant. After epinephrine no consistent changes were observed.

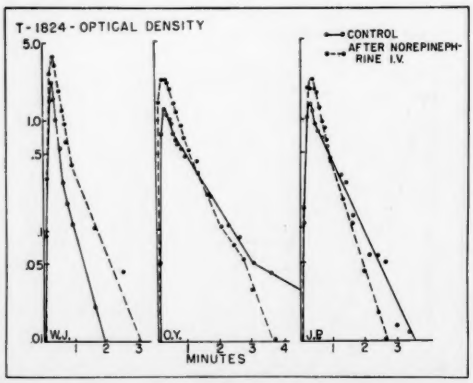

FIG. 3. EFFECTS OF SYSTEMICALLY ADMINISTERED NOREPINEPHRINE IN THREE SUBJECTS

Note increased dye concentrations and broadening of the curve around the peak.

Systemic norepinephrine and epinephrine

Epinephrine was infused at a rate of 10 to 22 μg. per minute until there was a significant elevation of systolic pressure. This was always accompanied by a detectable fall in diastolic blood pressure. The mean elevation of systolic pressure was 34 ± 8 mm. Hg, and the fall of diastolic pressure averaged 11 ± 7 mm. Hg. The average increase in mean [(systolic + diastolic)/2] pressure was only 11 ± 5 mm. Hg. The changes in mean circulation times, relative flows, and relative volumes in the two compartments failed to indicate any consistent pattern (Tables I and II).

Norepinephrine was infused at a rate to produce not only significant systolic but also diastolic hypertension and bradycardia. The average elevation of systolic pressure was 52 ± 18 mm. Hg and of diastolic pressure 30 ± 5 mm. Hg, the increase in "mean" arterial pressure averaging 42 ± 11 mm. Hg. Thus, the elevation of "mean" arterial pressure was approximately four times as great after norepinephrine as after epinephrine. The mean decrease in heart rate was 17 ± 7 beats per minute.

In contrast to the insignificant effects of epinephrine there was a significant decrease in T and increase in f_1/f_2 when the six cases with controls were compared on an intragroup basis (Table III). The ratio f_1/f_2 increased in all of the cases who had their own controls (Table I), the mean increase being 375 per cent over the control and the range 94 to 755 per cent. Although t_1 and t_2

TABLE II—*Continued*

differences between means under the various experimental conditions

t_2					f_1/f_2					v_1/v_2				
		Difference between means					Difference between means					Difference between means		
Mean	S.D.	Difference	S.D. of diff.	t	Mean	S.D.	Difference	S.D. of diff.	t	Mean	S.D.	Difference	S.D. of diff.	t
125	44				1.6	1.7				0.5	0.7			
68	60	57	15	3.7	34	58	32	9	3.6	10.7	19.5	10	4	2.8
370	172	245	8	31.0	1.2	0.9	0.4	0.6	0.7	0.2	0.2	0.3	0.16	1.8
108	22	17	22	0.8	1.8	0.5	0.2	0.75	0.2	0.4	0.4	1.0	0.21	0.4
150	45	25	25	1.0	1.3	0.6	0.3	0.4	0.8	0.3	0.14	0.2	0.17	1.1
139	80	14	28	0.5	1.4	0.6	0.2	0.7	0.3	0.25	0.06	0.25	0.2	1.3
125	12	0.2	25	0.004	6.7	5.5	5	2.4	2.2	1.8	1.8	1.3	0.8	1.6

were not significantly changed from the controls they both increased in relation to the decrease in T. Inspection of the curves disclosed a uniform change characterized by a broadening of the curve with increased dye concentrations around the peak. The junction or transition zone between t_1 and t_2 was shifted to the right (Figure 3). Thus, norepinephrine given systemically in dosages sufficient to produce a significant elevation of "mean" arterial pressure, including diastolic pressure, seemed to produce a change in the distribution of blood flow so that a larger proportion was carried in the rapid component. This change apparently did not occur when norepinephrine was given locally nor when epinephrine was given locally or systemically in dosages to produce a systolic but not diastolic hypertension.

DISCUSSION

These studies demonstrate that the biphasic pattern of blood flow in the forearm is subject to dynamic changes. When total blood flow was augmented by any one of several means (local exercise, intra-arterial tolazoline, reactive hyperemia) flow in the rapid component increased relative to that in the slow component. The increase in flow of the rapid component was accompanied by an increase in the relative vascular volume of the rapid component. These data represent an extension of the previous observations on a group of subjects studied under resting conditions. In that series, f_1/f_2 and v_1/v_2 were found to be inversely related to T (1).

Whereas the ratios f_1/f_2 and v_1/v_2 could be increased by various maneuvers, no means was found to significantly reduce them. Even venous congestion, which resulted in marked prolongation of the mean circulation times, probably reflecting a reduction in total blood flow (4), failed to produce a significant decrease in relative flow in the rapid as compared to the slow component. The local (intra-arterial) infusion of epinephrine and norepinephrine also failed to change the relationship between flows or volumes in the rapid as compared to the slow components.

It must be admitted, however, that the number of observations was not sufficient to rule out the possibility of minor changes in the measured parameters. In addition, the method of statistical analysis was more apt to underestimate than overestimate significant changes. Nevertheless, there were three cases with paired controls given norepinephrine intra-arterially and four given epinephrine. Inspection of these data fails to suggest the obvious and striking changes seen in other groups (Table I).

Epinephrine continuously infused intra-arterially produces no change in forearm blood flow while sustained intravenous infusion is followed by a slight vasodilation (5). Because of these opposing

TABLE III

Statistical analysis of data on six subjects given norepinephrine systemically, compared with their own controls

Parameter	Mean difference between means ± standard error	t
T	19.7 ± 6.2	3.2*
t_1	0.5 ± 1.45	0.3
t_2	4.5 ± 9.7	0.5
f_1/f_2	5.9 ± 2.0	2.9*
v_1/v_2	1.2 ± 0.9	1.3

* Significant at the 5 per cent level.

actions, the total forearm flow representing both skin and muscle may not be significantly changed. Norepinephrine, on the other hand, is said to produce slight decreases in forearm or calf blood flow (6) which were somewhat more prominent after intra-arterial infusion (7). This constrictor effect may have accounted for the slight increase in T following intra-arterial norepinephrine. However, the failure to reduce the flow and volume ratios in the rapid as compared to the slow component by any of the various experimental maneuvers, including intra-arterial norepinephrine and venous congestion, suggests that in the normal resting state the ratios f_1/f_2 and v_1/v_2 approach their minimal values; and whereas these ratios can be made to increase considerably under various stresses, apparently they cannot easily be reduced.

A surprising observation was that flow in the rapid component increased relative to that in the slow component following systemic norepinephrine infusion. The increase in dye concentration following systemic norepinephrine indicates a reduction in total forearm blood flow or vascular volume, or both. The broadening out of the early portion of the curves also suggests this. However, the earlier "disappearance" time observed in five out of six cases suggests that the predominant effect was on vascular volume rather than on blood flow since a decrease in flow without a volume change would tend to prolong rather than shorten the disappearance time. Obviously, the elevated arterial pressure would also tend to counteract a possible decrease in blood flow. Plethysmographic measurements during norepinephrine infusions indicate only slight decreases in forearm blood flow (6). Thus, the characteristic change in the dye concentration curve following systemic norepinephrine seemed to be due to the combined effects of increased pressure and increased local vasoconstriction.

These observations are in keeping with the previous studies, indicating a decrease in peripheral vascular volume after norepinephrine (8). [The previous studies showed that epinephrine also had this activity in the dog, but it should be recalled that unlike man the hemodynamic effects of epinephrine in the dog resembled those of norepinephrine (9).] It was indicated that either sympathetic activity or norepinephrine reduces the volume of the small vessels, including postarteriolar vessels, with a resulting increase in venous return.

It is suggested that the present data may throw further light on this phenonmenon. A decrease in T and increase in the ratio f_1/f_2 could not occur as a result of arteriolar constriction alone, but must include postarteriolar narrowing as well. The failure of v_1/v_2 to change significantly indicates that both the rapid and slow components shared in the vasoconstriction. The net effect of such a decrease in peripheral vascular capacity, aided and abetted by an increase in arterial pressure, would be to increase the velocity of flow through the generally constricted peripheral vascular bed. The result would be more fast-moving and less slow-moving blood flow; that is, an increase in the ratio f_1/f_2.

SUMMARY AND CONCLUSIONS

Various experimental procedures were utilized to study their effects on the relationship between the rapid and slow components of blood flow in the human forearm (1). Local hyperemia produced by intra-arterial injection of tolazoline, reactive hyperemia, or local exercise increased the flow and volume in the rapid component relative to that in the slow component. Venous congestion resulted in marked but nearly proportionate prolongations of mean circulation times. The relative flows and volumes of the two components were not significantly changed. Local (intra-arterial) infusion of either epinephrine or norepinephrine and systemic administration of epinephrine produced no obvious changes in these parameters although the number of cases, especially those with paired controls, was insufficient to rule out small changes. Systemic infusion of norepinephrine produced characteristic changes in the time-concentration curve of the dye, indicating a relative increase in blood flow of the rapid component. It is suggested that the latter was due to the hypertension plus vasoconstriction in the forearm occurring in response to significant elevation of mean pressure.

These observations demonstrate that the biphasic system of forearm blood flow and volume is dynamic, the relative proportion of the rapid and slow components changing under appropriate stimuli. The data also suggest that under normal

resting conditions the ratios f_1/f_2 and v_1/v_2 are near the lower levels of their possible range.

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THE RELATIONSHIP OF SERUM HEXOSAMINE, GLOBULINS, AND ANTIBODIES TO EXPERIMENTAL AMYLOIDOSIS [1]

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Amyloid as a disease in human beings occurs not only *de novo,* but also secondary to a variety of chronic infectious diseases, to rheumatoid arthritis, and to multiple myeloma. These apparently unrelated diseases may, therefore, have some characteristics in common, as evidenced by their ability to produce amyloid. Knowledge of the specific biological alterations that are responsible for secondary amyloidosis may highlight certain factors common to these diverse primary diseases as well as aid in elucidating the basic physiological alterations responsible for the disease process of rheumatoid arthritis, which is complicated by a high incidence of amyloidosis.

Secondary amyloidosis has been produced in experimental animals by numerous workers (1–11). Whether the amyloid produced in experimental animals is identical in chemical structure and pathogenesis to the primary and secondary amyloid found in human beings has not been established. Though several workers have pointed out the frequency with which hyperglobulinemia occurs (2, 3, 7, 12, 13), others have demonstrated that alterations in blood lipoproteins (7) and elevation of the serum hexosamine (14) occur. On the basis of these observations it has been suggested that amyloid may be the product of an antigen-antibody precipitate (13, 15, 16) or the indirect result of other immunological reactions.

The purpose of this study was to produce secondary amyloidosis in a series of rabbits in order to 1) examine and relate the biochemical and immunological derangements that occurred during the development of amyloidosis, and 2) compare the chemical composition of amyloid with that found in human beings. Results of the studies of the chemical identity of these types of amyloid will appear in a separate report. The observations reported here demonstrate that elevation of serum globulins and, particularly, of the serum protein bound hexosamine occurs in all animals developing amyloidosis, whereas there was no correlation between the precipitable antibody titer and the degree of amyloidosis.

MATERIAL AND METHODS

Plan of study. The study was divided into two parts, performed during successive years. In the initial study (A), a group of 12 rabbits, of the same strain of New Zealand whites, and weighing approximately 3 Kg. each, was subjected to biweekly subcutaneous injections of 5 ml. of 10 per cent casein suspended in water, to which penicillin was added in a dose of 1,000 units per ml. The suspension was injected subcutaneously into the back of the rabbit, a different site being selected for each injection. Three additional rabbits of the same strain, age and weight were kept as uninjected controls. The rabbits were bled periodically by intracardiac puncture, about 20 ml. being withdrawn each time. The blood was allowed to clot, and the serum was stored at $-20°$ C. until time of analysis.

Of the original group of 12 injected rabbits, four died at three months or less—one of infection, one with convulsions immediately following casein injection, one of nephritis, and one following intracardiac puncture. These rabbits were eliminated from the study; none displayed histologic evidence of amyloidosis at autopsy. The eight remaining rabbits received biweekly injections and were bled at two month intervals for 6 to 15 months. Five were sacrificed; three died—one (No. 18) with a severe groin abscess, and two (Nos. 12 and 13) shortly after casein injections. The clinical and histological data and the result of analyses of the serum obtained at the serial bleedings are recorded in Tables I and II, under Group A.

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TABLE I

Clinical and pathological observations of all rabbits in study

Rabbit no.	Sex	No. of injections	Months until death	Mode of death	Amyloid in spleen	Amyloid in kidney	Comments
				Group A			
Controls							
23	♀	0	15.5	Sacrificed	0	0	
24	♀	0	15	Sacrificed	0	0	
25	♀	0	15	Sacrificed	0	0	
10% casein twice weekly							
18	♀	42	6	Died	++++	±	Inguinal abscess
8	♀	82	11.5	Sacrificed	++++	±	Cortisone, last 8 days
14	♀	71	10.5	Sacrificed	++	++++	Peritonitis; severe glomerular and tubular damage
9	♀	100	15	Sacrificed	++	+	
17	♀	87	13	Sacrificed	+	++	Severe glomerular and tubular damage
13	♀	64	8.5	Died	+	0	Died after injection
11	♀	100	15	Sacrificed	+	0	
12	♀	92	13.5	Died	±	+	Severe nephritis; pulmonary intra-arterial hyaline deposits

Rabbit no.	Sex	No. of injections	Months until death	Mode of death	Time of biopsy (*months*)	Amyloid in spleen at biopsy	Amyloid at death: In spleen	Amyloid at death: In kidney	Degree of emaciation	Subcutaneous nodules
					Group B					
10% casein twice weekly										
67	♀	46	4	Spontaneous			+++	0	++	
69	♀	57	7	Sacrificed			++++	Minimal hyaline	+++	
58	♀	53	6⅓	Sacrificed	3.5	+ ++	++++	+++	+++	Yes
56	♀	37	6⅔	Found dead	3.5	++	++++	++	0	Yes
55	♀	59	7	Found dead	3.5	+	++++	+++ ? (Pyelonephritis)	++	No
59	♀	74	9	Sacrificed			+++	+++		
47	♂	60	6	Found dead	3.5	0	++	++	++ ? (Pyelonephritis)	No
60	♀	68	8¼	Sacrificed			++	+++	0	Yes
Single injection 10% casein										
61	♀	1	8	Sacrificed	3.5	0	+++	0 (Severe renal tubular damage)		

Following completion of this study, a second study (B) was commenced. One group of six rabbits was studied in an identical manner to those described above, except that prior to the first injection the animals were anesthetized with Nembutal® and their spleens were eased through a muscular incision and fixed subcutaneously at the lower rib margin.[4] An initial biopsy was obtained at this time. Repeat biopsy was obtained after three and one-half months by way of a cutaneous incision. Unfortunately, the adhesions were often so dense that it was felt that it would have been easier to re-biopsy the spleen in its natural location. These six rabbits, together with two additional rabbits whose spleens had not been transplanted, received serial injections of 10 per cent casein twice weekly. All of these animals developed amyloidosis and are included in Table I (Group B). In addition, three animals with spleen transplants received only one injection of casein. One (No. 61) developed massive amyloidosis and is included in Group B, Table I. The other two did not show signs of amyloidosis when sacrificed after 8 and 12 months, respectively, nor did they exhibit any of the chemical or immunological alterations noted in the animals with amyloidosis and so were not included in the series.

The animals were bled by intracardiac puncture before beginning the casein injections and at one to two month intervals until death. The serum was stored in the deep freeze. Sacrifice was performed by exsanguination. As soon as possible after death, each animal was examined

[4] This procedure was performed by Messrs. Bob Bulwinkel and Donovan A. Baumgartner of the Harvard Medical School.

TABLE II

Chemical and immunologic observations of all rabbits in study

Rabbit no.	Time in months	Serum concentration						
			Globulins					
		Alb.	Alpha	Beta	Gamma	Casein antibody	Hexosamine	NPN
				Group A				
Controls		*Gm. %*	*Gm. %*	*Gm. %*	*Gm. %*	*μg. N/ml.*	*mg. %*	*mg. %*
23	0	3.9	0.6	0.4	0.6	0	83	*
	8	4.5	0.6	0.5	0.6	0	66	
	12	4.0	0.5	0.5	0.5	0	66	
	13	4.3	0.6	0.5	0.4	0	79	
	15	4.5	0.6	0.7	0.7	0	91	39
24	0	5.2	0.9	0.7	0.7	0	85	46
	12	4.5	0.8	0.6	0.6	0	87	
	13	4.6	1.0	0.7	0.5	0	101	
	15	4.9	0.5	0.4	0.7	0	79	39
25	0	4.1	0.6	0.5	0.6	0	70	35
	12	3.9	0.5	0.5	0.6		62	
	13	4.1	0.6	0.4	0.7		70	
	15	4.0	0.5	0.7	0.6	0	88	30
Mean of controls		4.3±0.5 (N=37)†	0.64±0.15 (N=37)	0.58±0.13 (N=37)	0.81±0.3 (N=37)		77.3±8 (N=19)	
10% casein S.C. twice weekly								
18	0	4.6	0.7	0.5	0.7	0	82	
	2	3.2	0.8	0.7	2.1	710	129	39
	3	3.0	0.8	0.8	2.2	280	116	
	5	3.8	1.0	0.9	3.2	435	137	85
8	0	4.4	0.5	0.5	0.7	0	83	
	2	3.1	0.7	1.0	2.4	0	110	38
	3	4.3	0.8	0.9	1.9	175	124	
	5	4.0	0.5	0.4	1.3	0	83	
	8	3.8	0.6	0.7	1.4	175	112	38
	10	3.6	0.7	0.7	1.5	0	104	
	11	3.0	1.0	0.9	1.7	85	124	
	2 days‡	3.4	1.0	1.0	1.4	0	139	
	5 days	3.0	0.9	0.9	1.0	0	108	
	7 days	3.0	0.9	0.8	0.8	0	108	
	10 days	3.1	0.6	0.7	0.7	0	120	42
14	0	4.1	0.9	0.7	1.1	0	81	
	2	4.0	1.0	0.9	2.0	40	118	39
	3	3.4	1.1	0.9	1.8	35	112	
	5	4.1	0.7	0.5	1.2	145	77	
	8	2.8	1.1	0.7	1.9	50	122	40
	10	1.0	1.3	0.5	1.5	185	110	
	11	1.7	1.5	0.7	2.3	0	124	96
9	0	4.3	0.6	0.6	1.0	0	73	
	2	3.1	0.7	0.8	1.9	0	91	
	3	3.0	0.8	0.6	1.4	285	112	35
	5	3.7	0.7	0.6	1.5	0	79	
	8	3.5	0.8	0.6	1.9	0	91	
	10	3.8	0.6	0.5	2.6	0	96	
17	0	4.2	0.5	0.6	0.5	0	62	
	2	4.0	0.7	0.6	1.2	0–trace	83	33
	3	3.0	0.9	0.9	1.0	0	104	
	5	3.4	0.6	0.8	1.0	0–trace	79	
	8	3.6	0.7	0.8	1.1	0	91	
	10	3.2	1.0	1.0	1.0	0–trace	104	66
	12	1.2	1.1	0.9	0.9	0	100	
	13	1.9	1.3	0.8	0.8	0–trace	112	86

* Blank indicates not measured.
† N includes the control or pre-injection values taken on many treated animals.
‡ Days of cortisone injection.

TABLE II—*Continued*

Rabbit no.	Time in months	Serum concentration						
		Alb.	Globulins			Casein antibody	Hexosamine	NPN
			Alpha	Beta	Gamma			
		Group A—*Continued*						
		Gm. %	*Gm. %*	*Gm. %*	*Gm. %*	*μg. N/ml.*	*mg. %*	*mg. %*
13	0	4.1	0.8	0.6	1.7	0	89	
	2	3.6	0.8	0.8	1.6	0	97	
	3	3.4	1.0	0.8	1.3	0	89	
	5	4.7	1.0	0.9	2.0	trace	104	59
	8	3.6	0.9	0.6	1.7	0	97	
	9	4.1	0.6	0.5	1.3	0	89	45
11	0	5.4	0.4	0.4	0.6	0	66	
	2	3.7	0.6	0.7	1.8	0	99	
	3	3.2	0.5	0.5	1.3	0–trace	108	37
	5	4.5	0.7	0.6	1.4	0	83	
	8	4.1	0.7	0.8	1.6	55	83	
	10	4.4	0.7	0.7	1.4	0	91	
	12	4.0	0.7	0.8	1.3	10	108	39
	13	4.1	0.8	0.9	1.4	0	99	
	14	4.3	0.8	0.7	1.4	0		
	15	4.9	0.4	0.5	1.0	0	91	39
12	0	3.6	0.5	0.5	1.2	0	79	
	2	3.8	0.8	0.7	1.9	0	104	
	3	3.4	0.5	0.5	1.4	trace	89	37
	5	4.0	0.6	0.5	1.4	0	83	
	8	4.0	0.6	0.7	2.2	0	75	
	10	3.8	0.6	0.7	1.4	trace	87	51
	12	2.8	0.6	0.7	1.4	0	99	
	13	1.8	0.9	0.9	1.2	trace	99	43
				Group B				
10% casein S.C. twice weekly						§		
67	0	2.5	0.8	1.1	1.1	0	102	31
	2	2.4	0.7	0.6	1.1		92	
	4	2.4	0.4	1.0	1.2	1	164	33
69	0	4.1	0.5	0.5	0.7	0	77	
	2	2.6	0.6	0.7	1.9		130	
	4	2.1	1.0	0.8	2.3	6	118	
	6	2.4	0.8	0.9	2.1	9	145	
58	0	3.1	0.4	0.4	0.4	0	75	28
	2	4.4	0.8	0.8	1.9	11	120	38
	3	2.6	0.6	1.0	1.6	4	131	48
	5	2.0	0.6	1.1	2.5	8	141	22
	6	1.5	1.1	1.1	2.1	1	142	63
56	0	4.1	0.5	0.3	0.9	0	90	41
	2	3.9	0.5	0.4	1.4	4	110	56
	3	2.8	0.8	0.5	1.1	5	108	42
	5	1.7	0.8	0.8	1.0	1	110	38
55	0	3.4	0.4	0.4	0.6	0	83	42
	2	3.2	0.6	0.6	1.4	11	120	41
	3	3.2	0.6	0.6	1.4	6	115	42
	5	2.7	0.7	0.7	0.7	9	150	34
	7	1.4	1.2	0.9	1.3	0	148	
59	0	3.3	0.5	0.5	0.5	0	73	45
	2	3.1	0.5	0.7	1.5	16	116	38
	3	3.1	0.8	0.5	1.4	9	110	41
	5	3.1	0.6	0.6	1.9	16	121	42
	7	2.4	0.7	0.6	2.8	8	123	38
	9	1.9	1.0	1.4	1.5	3	134	119

§ Number of antigen additions.

TABLE II—*Continued*

Rabbit no.	Time in months	Serum concentration						
		Alb.	Globulins			Casein antibody	Hexosamine	NPN
			Alpha	Beta	Gamma			
		Group B—*Continued*						
		Gm. %	*Gm. %*	*Gm. %*	*Gm. %*	§	*mg. %*	*mg. %*
47	0	3.1	0.4	0.5	0.3	0	95	47
	2	3.7	0.7	0.7	1.1	0	126	38
	3	3.2	0.6	0.7	0.7	0	123	37
	5	3.1	0.7	0.7	0.9	0	128	17
	7	1.4	1.1	0.9	0.5	0	135	38
60	0	4.1	0.5	0.6	0.6	0	80	39
	2	3.2	0.6	0.7	1.3	8	98	39
	5	3.0	0.6	0.9	1.3	4	98	28
	7	2.6	0.7	0.9	1.6	5	112	37
	8	2.3	1.0	1.1	1.1	0	115	
Single injection 10% casein								
61	0	3.5	0.4	0.3	0.6	0	72	33
	2	4.2	0.6	0.6	1.4	0	97	35
	3	3.8	1.0	0.9	2.2	0	122	41
	5	4.1	0.7	0.7	2.4	0	121	
	7	3.9	0.6	0.7	2.6	4	121	25
	8	3.0	0.7	0.7	2.1	1	139	33

§ Number of antigen additions.

and blocks of tissues were fixed in formaldehyde with $CaCl_2$. Sections were prepared and stained with hematoxylin and eosin, congo red and methyl violet. The liver, spleen and kidney of each animal, as well as subcutaneous nodules, heart and lungs, if they appeared abnormal, were stored in sealed bottles in the deep freeze.

Chemical methods. The total protein of the serum was measured by the $CuSO_4$ method (17) with frequent checks by the gradient density method (18). Serum electrophoresis was obtained by the free-hanging "horizontal strip" method (19) on Whatman No. 1 paper at 22° C., at 100 volts, 1.5 ma, for 16 hours in veronal buffer (pH 8.6, 0.01 μ). Following staining for proteins with bromphenol blue, the paper strips were cut and each protein fraction eluted with 0.1 N NaOH and the optical density determined at 575 mμ. From this figure and the total protein the amount of protein in each fraction was calculated. The serum hexosamine concentration was determined by the Elson and Morgan reaction as modified by Boas (20). The antibody nitrogen of the casein-antibody precipitate was determined by the Kjeldahl method (21).

Estimations of circulating antibody concentrations were obtained by two different methods. Rabbits of Group A were initially studied by the following method: Following thawing at room temperature, the serum was distributed in 0.5 ml. aliquots into each of a series of test tubes. To each tube was added 0.5 ml. of saline solutions of casein with nitrogen concentration of 1,800, 250, 125, 62.5, 31 and 7.7 micrograms per ml., respectively. After incubation for 30 minutes at 37° C., the mixtures were allowed to stand overnight at 0° C., centrifuged, and the precipitates were graded. The supernatant fluids were tested for antigen and antibody excess and the nitrogen values of the antigen-antibody precipitates obtained in the region of slight antigen excess were obtained by the Kjeldahl method (21). From this figure, the antibody nitrogen concentration of the respective serum was calculated and the maximum values for each serum are recorded in Table II.

In Group B rabbits, an estimation of the concentration of antibody was obtained in a different manner. To 1 ml. of serum, 0.25 ml. aliquots of casein solution (18 gamma N per ml.) were added serially on successive mornings. After each addition the mixture was incubated at 37° C. for 1 hour and then chilled at 0° C. for approximately 24 hours; the precipitate was centrifuged off and a new 0.25 ml. aliquot was then added. This procedure was continued until the addition of antigen failed to yield any precipitate. The number of additions which did yield a precipitate were recorded as an index of the titer of precipitating antibody in that specimen. Simultaneous quantitative determination of the amount of antibody nitrogen obtained in a manner similar to that employed in the Group A rabbits indicated, in several sera studied, that each addition of antigen, containing 4.5 gamma of nitrogen, resulted in the precipitation of approximately 45 gamma of antibody nitrogen.

The specificity of the antigen-antibody precipitation reaction was established by the demonstration that no reaction occurred on cross-reacting casein with anti-bovine plasma albumin rabbit serum, or by reacting bovine plasma albumin (Armour) with anti-casein rabbit serum.

Serum nonprotein nitrogen values, serum uronic acid (22), cryoglobulins (23), and serum cholesterol values were obtained by standard methods. Paper chromato-

graphic identification of serum hexosamine was done by a method previously outlined (24). The antigen antibody reaction was studied by the agar diffusion method of Ouchterlony (25).

Radioactive methods. In three casein injected rabbits (59, 60, 61) from series B, an attempt was made to determine whether the administered casein was being deposited in the spleens as amyloid. Two normal rabbits served as controls. Approximately 0.2 ml. of a solution of I^{131} labeled sodium caseinate, 8 mg. per ml., (100 μc), prepared in this laboratory by a modification of the method of Pressman and Eisen (26), was injected subcutaneously as a tracer. The radioactivity of the subcutaneously implanted spleen, of the underlying body wall (from which the spleen had been deflected), and of the original site of injection of labeled casein was then determined at intervals for 24 hours by means of a directional counter. Once the time of maximum uptake by the spleen had been determined (between 4 and 10 hours after injection), a ten-times larger dose of labeled caseinate was administered subcutaneously in multiple sites and the animal was sacrificed after this interval. Aliquots of spleen, kidneys, heart, striated muscle, liver, stomach wall and serum were obtained and determinations of wet weight and radioactivity were obtained, by both direct counting and by radioautography. In two rabbits (No. 60 and one control) radioactive labeled casein was administered daily for 4 days prior to sacrifice, 10 hours after the last injection. Sections of the spleen and kidney were studied radioautographically, and the tissues were weighed and counted as usual.

RESULTS

A. Clinical and pathological

The overall incidence of amyloid disease in the rabbits studied, as indicated by biopsy and autopsy, is shown in Table I, in which they are listed in general order of the amount and rapidity of amyloid formation. None of the three control rabbits or of the four which died in less than four months demonstrated amyloid accumulation in any of the organs studied. Repeat biopsy of the spleens of five rabbits in Group B after three and one-half months of injections revealed minimal but definite traces of amyloid in three (Nos. 55, 56 and 58). All of these rabbits went on to display massive amyloidosis at death three and one-half months later. Of the three rabbits which received only one injection of casein, two were free of amyloidosis both at biopsy at three and one-half months and at autopsy five or more months later; one, however (No. 61) exhibited a moderate accumulation in the spleen at time of death.

The histologic appearance of the amyloid displayed a consistent pattern in both groups of rabbits. The earliest visible change in the spleen was the accumulation, especially in perifollicular areas, of small masses of hyaline material, which stained with congo red. In some animals the material was widespread throughout the loose interstitial tissue, as well as in the follicular areas. In others, massive extracellular accumulation appeared in the perifollicular areas, leaving the interstitial tissue largely uninvolved. Finally, in several rabbits the spleen became almost totally replaced, only sparing a few trabeculae and lymphoid follicles. The most common renal abnormality was the accumulation of amyloid substance in the glomeruli, sometimes diffusely along the basement membrane, but occasionally appearing to be engulfed in macrophages. In many of the rabbits the glomeruli were largely or totally replaced by amyloid. In some instances, small amounts of amyloid were also present in the interstitial areas around the collecting tubules. In several rabbits, notably Nos. 47, 56 and 55, there were focal accumulations of polymorphonuclear lymphocytes in the interstitial areas, suggesting pyelonephritis. The livers were usually normal, except for some central hyalinization. In all of the instances noted above, the amyloid stained, at least to some degree, with congo red.

Histologic examination of the heart and skeletal muscles was negative. The nodules present at sites of injection consisted of masses of eosinophilic granular material superficially resembling amyloid but on closer examination, were found to exhibit the appearance of necrotic material and cellular debris and did not stain with congo red. This amorphous material was surrounded by an area of inflammation with infiltration of polymorphonuclear cells.

The clinical course of the rabbits was reviewed in an attempt to identify the characteristics which were manifest by the rabbits which developed severe amyloid disease. There appeared to be no correlation between the distribution of amyloid, degree of organ involvement and the duration of the injections. In six of the rabbits (Nos. 14, 9, 11, 58, 56 and 60) pea-sized nodules developed at the site of many of the injections, containing thick creamy material having the histologic characteristics mentioned above. The material from one of these yielded a heavy growth of *H. influenzae* on

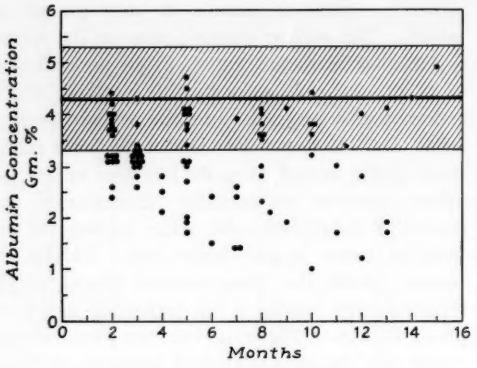

FIG. 1. SERUM ALBUMIN

The serum albumin concentration of each rabbit developing amyloidosis is plotted against the months of casein injection. The shaded area represents two standard deviations on either side of the mean of normal values.

culture, whereas several others were sterile. There was no relationship between the presence of these nodules and the amount of amyloid. On the other hand, one rabbit (No. 18) which developed the most massive deposits of amyloid in the shortest period of time had developed, shortly before death, a massive inguinal abscess. Emaciation was conspicuous in several of the rabbits, particularly in those with severe renal damage and azotemia.

Only in those animals which died during the first few months of injections, and which did not show amyloid disease, did anaphylaxis appear to be a cause of death. One of these had widespread pulmonary intravascular hyaline deposits, as did one of the animals developing amyloidosis (No. 12). Two animals, one that developed (No. 8) and one that did not develop amyloid, received 25 mg. of cortisone intramuscularly daily for one week prior to death; although the cortisone appeared to hasten the development of cachexia and death, no conclusion could be drawn as to its effect upon the development of amyloidosis.

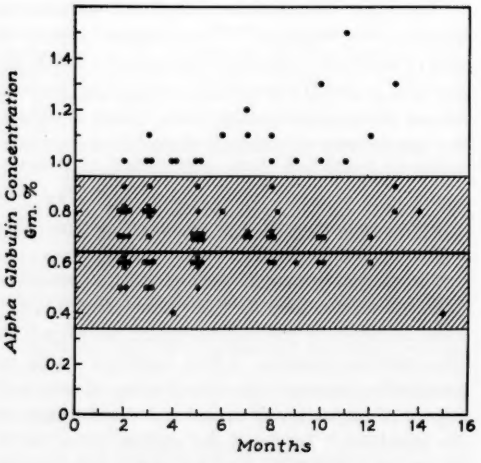

FIG. 2. ALPHA GLOBULIN

The serum alpha globulin concentration of each rabbit developing amyloidosis is plotted against the months of casein injection. The shaded area represents two standard deviations on either side of the mean of normal values.

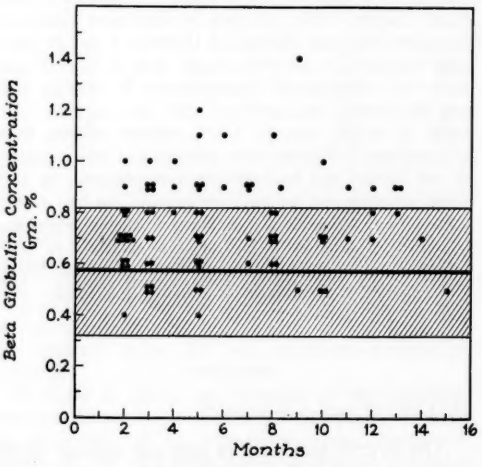

FIG. 3. BETA GLOBULIN

The serum beta globulin of each rabbit developing amyloidosis is plotted against the months of casein injection. The shaded area represents two standard deviations on either side of the mean of normal values.

B. Chemical

The results of the chemical determinations performed on the sera of these rabbits are recorded in Table II.

Electrophoresis of serum. A decrease from normal in the albumin values as determined by paper electrophoresis exceeding two standard deviations was demonstrated at some time in 17 of 18 of the animals developing amyloid (Figure 1). Elevations of the alpha globulins exceeding two stand-

ard deviations occurred in 13 rabbits (Figure 2). Equally significant deviations of the beta globulins occurred in 15 animals and of the gamma globulins in 13 (Figures 3 and 4). The most marked change in albumin concentration was noted after 10 to 12 months in those rabbits which developed renal disease and azotemia. There was a rough correlation between the amount of hyperglobulinemia and the amount of amyloid (Figure 5).

Serum hexosamine. In nearly all animals receiving serial injections of casein the serum hexosamine concentration had risen two standard deviations within two months (Figure 6), and as a rule it remained persistently elevated throughout

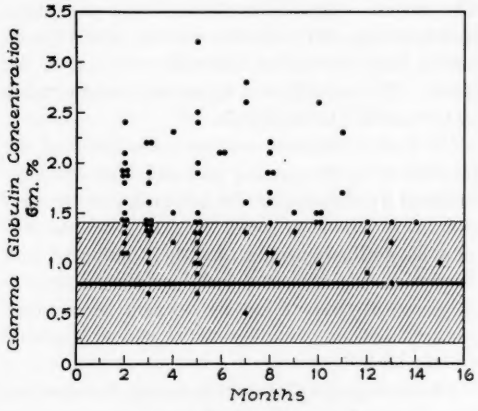

FIG. 4. GAMMA GLOBULIN

The serum gamma globulin of each rabbit developing amyloidosis is plotted against the months of casein injection. The shaded area represents two standard deviations on either side of the mean of normal values.

the course of the injections. There also appeared to be a correlation between the severity of the amyloid disease and the increase in hexosamine concentration during the period of injections (Figure 5).

Antibody nitrogen. The precipitable casein antibody nitrogen concentration in the rabbit serum exhibited no definite pattern. Of the eight animals in Group A, two demonstrated high antibody titers (200 to 600 micrograms antibody N per ml.), three a medium titer (100 to 200 micrograms Ab N per ml.), and three either none or a very low titer (0 to 100 micrograms Ab N per ml.). The highest titer appeared between the

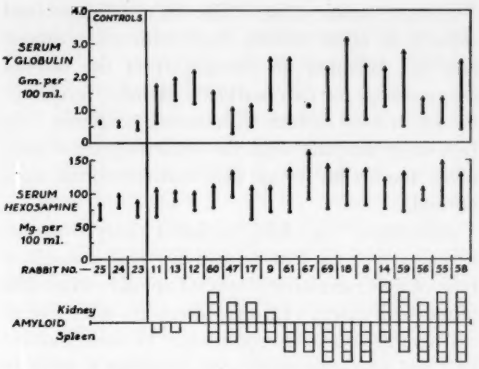
CONTROLS
SERUM γ GLOBULIN
Gm. per 100 ml.
SERUM HEXOSAMINE
Mg. per 100 ml.
RABBIT NO. 25 24 23 11 13 12 60 47 17 9 61 67 69 18 8 14 59 56 55 58
Kidney
AMYLOID
Spleen

FIG. 5. RELATIONSHIP OF AMYLOID HEXOSAMINE AND GAMMA GLOBULIN

The circle at the base of the arrows is the initial concentration and the tip of the arrow is the highest concentration reached during development of amyloidosis. Each block represents an increment (one plus) of amyloid infiltration of either spleen or kidney.

third and fifth months; usually there was a decrease in titer before death, particularly when associated with cachexia and renal disease. There appeared to be no correlation between the severity of amyloidosis and the antibody titer. The titers of precipitable casein antibody in Group B were in all respects similar to those of Group A (Table II).

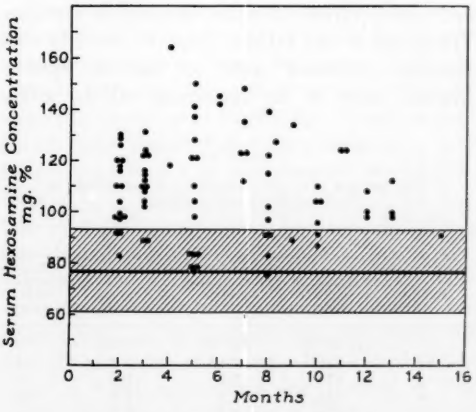

FIG. 6. SERUM HEXOSAMINE

The serum hexosamine concentration of each rabbit developing amyloidosis is plotted against the months of casein injection. The shaded area represents two standard deviations on either side of the mean of normal values.

Serum uronic acid. The values determined serially in eight rabbits, both with and without amyloid, revealed no changes from the normal range except for two markedly elevated levels obtained in two rabbits with severe azotemia. In two other animals with the same degree of azotemia the serum uronic acid concentrations were normal.

Skin tests. In order to detect delayed or tuberculin type of hypersensitivity and immediate type of hypersensitivity, several animals were skin tested with casein (1.2 mg. nitrogen) after five to six months of casein injections. Definite induration and erythema developed, reaching a peak in 4 to 6 hours, and subsiding within 24 hours. There was no reaction of the delayed type, nor did control animals give any reaction.

Serum nonprotein nitrogen (NPN) and cholesterol and lipemia. Serum nonprotein nitrogen concentrations were performed during the course of the study and at the last bleeding on each rabbit in both groups. Five of the seventeen rabbits (Nos. 14, 17, 18, 58, 59) developing amyloid disease showed marked elevations at the last bleeding; each of these rabbits had histologically demonstrable renal damage. Estimations of serum cholesterol, performed in two of the animals with azotemia (Nos. 14 and 17), showed elevated concentrations in both. Normal cholesterol concentrations were obtained in animals which had normal concentrations of blood nonprotein nitrogen. The serum of two rabbits (Nos. 14 and 18) with azotemia exhibited mild to marked lipemia, whereas none of the remaining rabbits, either control or receiving injections, demonstrated lipemia. It seems likely that the disturbance in cholesterol and fat metabolism was related to the renal disease rather than to the amyloidosis *per se.*

Serum cryoglobulins. None were demonstrated during the course of these observations.

Antigen-antibody precipitate. The concentration of hexosamine in twice washed casein-antibody precipitates from the sera of two rabbits that developed amyloidosis was 1 and 1.2 per cent of the precipitating protein. The quantity of hexosamine in the precipitate constituted less than 5 per cent of the total serum hexosamine and thus could not account for any significant amount of the elevated serum hexosamine concentration. The amount of hexosamine found in the precipitate corresponded well with that amount which disappeared from the serum following addition of the casein. The contribution to the hexosamine value by the casein was negligible.

Of further interest was an estimation of the proportion of the gamma globulin that was precipitated by the casein. In six instances the precipitable antibody nitrogen was converted to precipitable antibody protein, and then compared with the gamma globulin as determined by the electrophoresis. Between 6 and 24 per cent (mean, 16.8 per cent) of the gamma globulin was precipitated (Table III).

Chromatography of rabbit serum hexosamine. Because the hexosamine in human amyloid tissue appears to be both glucosamine and galactosamine in a ratio of approximately 4:1 (27), it was of interest to study the sera of amyloid rabbits in order to determine whether this same ratio was present. No galactosamine was demonstrable, however, in the sera by paper chromatography. In sera from three rabbits, hexosamine was determined before and after dialysis against isotonic saline (pH 7). In no case did the concentration decrease.

Agar diffusion antigen-antibody studies. The rabbit serum antibodies to the casein used in the production of amyloid were studied by the Ouchterlony agar diffusion technique (25). Against that casein four distinct bands were noted in the agar. Furthermore, the serial bleedings from a given animal showed changing intensity of the different bands during the course of the injections. Casein is known to have three electrophoretic

TABLE III

The percentage of precipitable casein-antibody in rabbit gamma globulin

Rabbit no.	Casein precipitable Ab titer	Casein precipitable protein (Ab ×6.25)	Gamma globulin	Gamma globulin precipitable by casein
	μg. N/ml.	*mg./ml.*	*Gm. %*	*%*
18	710	4.45	2.1	21
8	175	1.1	1.9	6
9	285	1.8	1.4	13
22*	570	3.55	1.5	24
4	768	4.8	2.5	19
(1 wk. later)	520	3.25	1.8	18

* Animal No. 22 received 1 per cent casein in Amphogel® five times weekly and is included only in this small study, being part of another study and report.

components. The presence, however, of four agar diffusion bands suggested that impurities in the casein had served as antigens. Therefore, these same sera were also tested against casein [5] that had been reprecipitated several times, but the same number and intensity of agar diffusion bands resulted. Sera from control animals gave no bands.

Radioactive studies. The rates of declining radioactivity at the sites of injection of I^{131} labeled casein and of increasing activity over the parenchymal organs were similar in all rabbits studied, whether or not they had received serial injections of casein. The radioactivity at the site of injection fell to less than 40 per cent of its initial value within 3 hours and reached between 5 and 15 per cent of its initial value in 10 hours. There was no difference in radioactivity of the spleen and of the body tissues immediately beneath the spleen at any time following the injection, either in the control or the injected animals. This activity reached its peak between 4 and 10 hours after the time of injection, the time curves showing no consistent difference between the two groups. No significant uptake of radioactive material was noticed in the region of the thyroid gland.

Upon sacrificing the animal no significant differences in radioactivity were noted between tissues which were laden with amyloid and the relatively normal organs. Radioautographic studies showed no concentration of labeled material in the areas of amyloid deposits. It was concluded that no uptake of I^{131} labeled casein by the amyloid tissue could be demonstrated by this method.

DISCUSSION

It must be pointed out at the outset that there is no proof that so-called amyloidosis in experimental animals is the same disease as amyloidosis in human beings. On the other hand, the histological appearance of the deposits, including staining reactions, is similar in both conditions; the course of the disease is parallel and both in human beings and in experimental animals the disease is prone to develop in situations resulting in prolonged taxation of the defense mechanisms of the host. Indeed, except for the fact that experimental amyloidosis, in rabbits at least, seldom involves the liver to any great extent, the two conditions are quite comparable. Since, in the last analysis no one knows the nature or cause of human amyloidosis, attempts to gain perspective by considering so analagous an experimental disorder do not seem inappropriate.

It is apparent from the above study that biweekly subcutaneous injection of sodium caseinate (0.5 Gm.) is an effective method of inducing amyloidosis in rabbits. Of 14 animals receiving serial injections and surviving for four months or more, all exhibited the disease to some degree. Individual susceptibility appeared to be a greater determinant than duration of injections. Thus, three of the rabbits studied by biopsy exhibited moderated amyloidosis after three and one-half months of injections and displayed massive amyloid deposits by the time of sacrifice, three and one-half months later. On the other hand, one animal (No. 11) displayed only minimal evidence of the disease after a 15 month course of injections totalling 64 Gm. of casein.

Because one of the rabbits with severe amyloidosis in this series (No. 18) had a secondary infection, it is suggested that secondary infection may predispose the animal to more severe disease. On the other hand, in a subsequent study, culture of both the casein preparations used for injection and the subcutaneous nodules were sterile, indicating that secondary infection is neither frequent nor necessary in the development of amyloidosis by casein injections.

The studies on our rabbits indicated that many but not all of those animals which exhibited hypergammaglobulinemia during the course of the injections were the ones which developed massive amyloid deposits (see Figure 5). Other investigators have noted that hyperglobulinemia is a frequent antecedent to experimental amyloidosis, induced by a variety of agents (*2*, 3, *7*, 12, 13). The nature of the association between hyperglobulinemia and amyloidosis is unknown.

Eklund and Reimann (3) commented not only upon the fact that hyperglobulinemia preceded the development of amyloidosis, but also upon the hypoalbuminemia appearing after the development of renal amyloidosis and azotemia. Our own studies likewise indicate that only those rabbits developing azotemia, as a terminal complication of amyloidosis, exhibited a decrease in albumin concentration. The hypercholesterolemia and lipemia, the marked rise in serum uronic acid concentration, and the terminal fall in serum albumin

[5] Obtained from Dr. David Waugh, Massachusetts Institute of Technology, Cambridge, Mass.

concentrations and in casein antibody titer seemed to be dependent upon the development of renal insufficiency and are probably not related to the pathogenesis of amyloidosis.

Elevation of the serum hexosamine has been reported in one instance of human amyloidosis (14) as well as in a wide variety of other diseases and during stress in animals. In addition, amyloid-containing tissues have been shown to contain a higher concentration of hexosamine than normal tissues (27, 28). In this study there appeared to be significant correlation between the degree of elevation of the serum hexosamine and the amount of amyloid present at autopsy (Figure 5). Further studies of the serum hexosamine indicate that it is nondialyzable, presumably protein bound, and predominantly or entirely glucosamine. Inasmuch as the serum uronic acid concentration did not increase in parallel fashion, it can be presumed that the glucosamine was probably not in the form of hyaluronic acid or another acid mucopolysaccharide. These data, therefore, suggest that the serum hexosamine component may have been glycoprotein in nature distinct from the antibodies precipitable by casein.

The demonstration of precipitable antibodies to casein in the sera of rabbits developing amyloidosis in the study is of interest in several respects. Some of the animals developed within two months' time moderately high titers of antibody to the casein, while others developed minimal or undetectable amounts of antibody even after 15 months of casein injections. The absence of apparent relationship between the amount of circulating antibody and the degree of amyloidosis reflected by this data does not necessarily refute the concept of others (13, 15, 16) that amyloid represents a deposit of an antigen-antibody complex. Vazquez and Dixon (16), using fluorescent labeling techniques, have recently furnished the best evidence for this concept by detecting both gamma globulin and casein in the amyloid induced in rabbits by casein injections, though it must be pointed out that the gamma globulin and casein by this technique may constitute only a very small portion of the amyloid. It is entirely possible that the rapid precipitation of such a complex might have removed antibodies from the serum of our animals. The failure, however, to demonstrate I^{131} labeled casein in the amyloid of our rabbits leaves open the question as to whether amyloid is an antigen-antibody precipitate.

However, evidence is more conclusive that amyloid is not solely an accumulation of the antigen, *i.e.*, casein in this study. The presence of a significant hexosamine concentration in the amyloid of these animals (28) in contrast to the absence of hexosamine in casein is evidence that amyloid is not pure casein. Furthermore, one animal (No. 61) developed extensive amyloidosis after only one injection of casein (500 mg. total of casein). Finally, the failure of I^{131} labeled casein to be detected in the amyloid of these animals suggests that the amyloid was not pure casein.

Some peculiar antigenic properties of casein deserve comment. Although casein is known to have at least three electrophoretic components, the observation of four agar diffusion precipitin bands when testing the rabbit sera against casein, both that used for the injection and a reprecipitated preparation, suggests that a complex of these casein components (29) may serve as an antigen or the casein may in some fashion stimulate a heterogenous antibody. Such a complex antigen-antibody system may be difficult to evaluate by the quantitative precipitin technique used in the Group A animals, because the equivalence points may differ among these four antigen-antibody systems. Such difficulties in addition to the Danysz' phenomenon (30) may enter in the results obtained in the Group B animals.

The evidence presented above suggests that in these rabbits prolonged elevation in the concentration of serum globulins and hexosamine containing compounds may be associated with the eventual appearance of amyloid. Although no relationship was demonstrated between the serum precipitable antibody and the development of amyloidosis, there remains the definite possibility that nonprecipitating antibodies or other immunologic factors are intimately involved in the pathogenesis of amyloidosis.

CONCLUSIONS

1. Serial injections of sodium caseinate is a very effective, although slow and not entirely predictable, method of inducing massive deposits of amyloid in rabbits.

2. Elevation of all the globulins occurred both before and during the development of amyloidosis.

A fall in albumin concentration occurred in those animals which developed terminal renal insufficiency.

3. Elevation of the serum hexosamine concentration occurred throughout most of the course of injections in all of the rabbits which developed amyloidosis.

4. Significant titers of circulating antibodies could be demonstrated in many animals; while in general, the rabbits which exhibited the highest antibody concentrations developed large amyloid deposits, several animals developed definite deposits without having exhibited circulating antibodies.

ACKNOWLEDGMENT

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THE MEASUREMENT OF TISSUE ACID MUCOPOLYSACCHARIDES [1]

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Investigation of the metabolism of connective tissue acid mucopolysaccharides in normal and abnormal states has been hampered by a lack of practical analytical methods. Reported methods for measuring tissue acid mucopolysaccharides involve multiple steps of enzyme digestion and precipitation (1, 2), with consequent risk of loss of material as well as poor adaptability fo multiple analyses. This report describes a less complex procedure which gives reproducible results and good recovery of test material. Values are given for the acid mucopolysaccharide content of human dermis, subcutaneous tissue, aorta and thyroid gland, using both orcinol and carbazole reactions for uronic acid. Chromatographic and electrophoretic studies of the polysaccharides found and determination of the proportion digested by testicular hyaluronidase are also reported.

METHODS

Defatting and drying. Fresh or frozen tissue samples of 2 grams or less, cut into pieces about 2 mm. in diameter, were placed in 100 ml. of acetone on a wrist-action mechanical shaker for one hour. This was followed by additional one hour extractions with two 50 ml. quantities of acetone, and two 50 ml. quantities of diethyl ether. The tissue was then lyophilized overnight and stored in a desiccator.

Determination of total acid mucopolysaccharide content. 1. Weighed aliquots (75 to 200 mg.) of dried defatted tissue were extracted in 50 ml. of 0.5 N NaOH in a Waring blendor for 45 minutes in a cold room. The extraction procedure was stopped every 10 minutes to allow cooling of the container in an ice bath. The residual tissue was removed by centrifugation.

2. Ten ml. aliquots of the extract were placed in centrifuge tubes and brought to near neutrality by the addition of 0.5 ml. of 8.75 M acetic acid to each.

3. Three ml. of 17 per cent perchloric acid was then added to each tube to precipitate protein. After refrigeration at 4° C. for one hour to allow the precipitate to flocculate, the tubes were centrifuged at 2,000 rpm for 20 minutes.

4. Ten ml. of each supernatant solution was dialyzed overnight against running cold tap water to remove the perchloric acid and salts.

5. The dialysis bags were emptied quantitatively and washed with water; 0.2 ml. of 1 per cent protamine sulfate was then added to each. After refrigeration for one-half hour at 4° C., they were spun in a Servall SS-1 angle centrifuge at approximately 3,000 rpm for 30 minutes.

6. The precipitate was dissolved in 2.2 ml. of 2 M acetate buffer of pH 5.0 and duplicate 1.0 ml. aliquots were taken for determination of uronic acid by the carbazole method of Dische (3), with slight modification as previously reported (4).

7. Using orcinol, a combined uronic acid and hexose determination was done on a second 10 ml. aliquot of each original extract, processed in similar fashion up to step 6, and dissolved in the same buffer. The procedure of Brown (5) was followed; optical densities were read at 520 mμ and 670 mμ. An equimolar mixture of galactose and mannose was used as standard solution in addition to glucuronolactone, and calculations were done algebraically, based on the amount of color contributed by each type of sugar at the two wave lengths read.

Effect of incubation with testicular hyaluronidase. A 25 ml. aliquot of each original NaOH extract, in a 40 ml. centrifuge tube, was brought to near neutrality by the addition of 1.3 ml. of 8.75 M acetic acid. Proteins were removed by centrifugation at approximately 2,000 rpm for 20 minutes after the addition of 1.7 ml. of 60 per cent perchloric acid, and refrigeration at 4° C. for 30 minutes. Twenty-five ml. of the supernatant solution was dialyzed overnight against running cold tap water. The solution was transferred quantitatively and the volume brought to exactly 31 ml. with water. Two aliquots of 10 ml. were taken, and buffer was added to a final concentration of 1.0 M acetate, pH 6.0, and 0.15 M NaCl. Approximately 30 turbidity reducing units (TRU) [3] of commercial testicular hyaluronidase (Worthington or General Biochemical) was added to each. After incubation for 15 hours at 37° C., another 30 TRU of testicu-

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[2] John and Mary R. Markle Scholar in Medical Science.

[3] Assayed by the method of DiFerrante (6), which gave considerably lower values than the standard turbidimetric method of Meyer (7), run simultaneously by Dr. Manuel Epstein of the Endocrine Laboratory of Wayne State University College of Medicine.

lar hyaluronidase was added and incubation was continued for 5 hours. One ml. of 60 per cent perchloric acid was then added to remove the hyaluronidase protein. After centrifugation for 20 minutes at 2,000 rpm, 12 ml. aliquots were dialyzed overnight against running cold tap water. Dialysis bags were then emptied quantitatively and 0.2 ml. of 1 per cent protamine sulfate was added. After refrigeration, carbazole and orcinol determinations were performed.[4]

Hexosamine determinations. These were done following hydrolysis in 2 N HCl at 100° C. for 15 hours; the HCl was then rapidly evaculated with a vacuum pump. The method of Boas (8) was then followed.

Chromatographic and electrophoretic studies. Aliquots of the 0.5 N NaOH extract were processed through step 4 as above; the dialyzed solutions were then lyophilized to dryness. The residue was dissolved in a small quantity of water and chromatographed on S and S 598 filter paper, descending, using the buffered ethanol and propanol solvent systems and staining procedure previously reported (4). Electrophoresis was performed in a horizontal vapor-chamber apparatus, using 0.05 M acetate buffer of pH 4.2 at 400 volts for 3 hours. Papers were oven dried, fixed and stained in the same fashion as the chromatograms. Standards used included chondroitin sulfate from bovine nasal cartilage, hyaluronate from human synovial fluid and cock's comb, and heparin from commercial sources.

RESULTS

Completeness of extraction of acid mucopolysaccharides, as estimated by determination of hexosamine remaining in the tissue fragments after NaOH treatment, exceeded 95 per cent.

Recovery of chondroitin sulfate added to 50 ml. of 0.5 N NaOH in a Waring blendor and carried through the entire method averaged 92.1 per cent (Table I). When the chondroitin sulfate was exposed to the NaOH for longer than one hour, recoveries tended to be lower. Acetic acid was added, therefore, as soon as the extraction could be considered complete. A drop of phenolphthalein was useful to insure that the pH of the solution was kept on the alkaline side of neutrality, to prevent precipitation of mucopolysaccharide.

[4] Following completion of the studies reported herein, it was found that the method could be further simplified without lowering recoveries or reproducibility. Two ml. of 8.75 N acetic acid and 3 ml. of 70 per cent perchloric acid were added to the entire 50 ml. of extract before removal from the Waring blendor. After 30 minutes at 4° C., the tissue residue and precipitate were removed by centrifugation. The supernatant solution was divided into 4 aliquots of 10 ml., which were dialyzed and then processed as described above from step 5.

TABLE I

Recovery of chondroitin sulfate added to 50 ml. of 0.5 N NaOH and carried through entire procedure

Amount added (as uronic acid)	Recovered
μg.	%
266	103
287	91.3
287	78.8
288	79.3
292	87.5
292	111
292	93.1
307	94.8
307	86.9
310	94.2
310	89.3
310	96.2
Average	92.1

Reproducibility. Repeated determinations on the same tissue gave variations of approximately plus or minus 7 per cent for the carbazole and orcinol determinations of the total acid mucopolysaccharide. After hyaluronidase incubation, a variation of approximately plus or minus 8 per cent was found.

Interference by hexose-containing compounds. A small quantity of hexose, usually around 5 μg., was found in the final protamine precipitates by the orcinol determination, indicating the absence of significant contamination with glycogen or glycoprotein. The optical density produced by hexose in each carbazole determination was rarely more than 5 per cent of the total, and never more than 10 per cent. Correction for this contamination was not considered advisable. The absorption spectrum of the final precipitate in the carbazole determination matched that of glucuronolactone.

Effects of hyaluronidase incubation. Incubation with hyaluronidase removed an average of 88 per cent of chondroitin sulfate introduced in amounts of 20 to 3,500 μg. under the conditions tested; 74 per cent or more was removed in 95 per cent of the tests. When smaller amounts of chondroitin sulfate were used as starting material, digestion was not as complete, presumably because of the very low substrate concentration. In the studies reported, more than 20 μg. of acid mucopolysaccharide was present in the actual incubation mixture, but not all the mucopolysaccharide present was hyaluronidase-labile. In addition, some of the tissue extracts might have

TABLE II

Acid mucopolysaccharide content of samples of human dermis *

Age	Sex	Total acid mucopolysaccharide			Mucopolysaccharide remaining after hyaluronidase digestion		
		Carbazole	Orcinol	Carbazole/ orcinol	Carbazole	Orcinol	Carbazole/ orcinol
48	M	64.6	80.4	0.80	47	80	0.59
51	F	89.2	102	0.87	35	72	0.49
65	M	90.0	145	0.62	38	97	0.39
70	F	102	110	0.93	38	68	0.56
71	M	69.4	102	0.68	35	78	0.45
79	M	111	140	0.79	35	87	0.63
82	F	47.6	76.1	0.63	41	74	0.55
		Average		0.76	Average		0.52

* Figures given are μg. of uronic acid in acid mucopolysaccharide per 100 mg. of dried, fat-free tissue found using carbazole and orcinol determinations. Two hundred mg. samples were used for extraction.

contained material with an inhibitory effect on the activity of the enzyme. The results reported can therefore be considered only an estimate of the minimal amount of mucopolysaccharide labile to testicular hyaluronidase.

Samples of chondroitin sulfate were digested with hyaluronidase according to the procedure described and then dialyzed against tap water. Carbazole determinations showed that over 85 per cent of the original material had been reduced to dialyzable units.

Acid mucopolysaccharide content of human tissues. Tissues were obtained at autopsy up to 15 hours after death. Skin and subcutaneous tissue were obtained from the upper thoracic region when there had been no recognizable disease involving that area. Subcutaneous tissue was carefully separated by sharp dissection and the epidermis was removed by scraping with a razor blade. The acid mucopolysaccharide content of samples of dermis are given in Table II, and of samples of subcutaneous tissue in Table III.

Segments of lower thoracic aorta were obtained which were free of more than minimal gross atherosclerosis. The acid mucopolysaccharide content of these aortas are reported in Table IV.

Thyroid glands were obtained at autopsy and following surgical removal. The acid mucopolysaccharide levels obtained are reported in Table V.

Qualitative studies. To establish the mucopolysaccharide nature of the uronic acid found, the ratio of hexosamine to uronic acid (carbazole method) in the final protamine precipitate was determined for each tissue. Almost all of the ratios fell between 0.7 and 1.2, averaging approximately one for each of the tissues studied.

Between one-third and two-thirds of the acid mucopolysaccharide present in the extracts of the

TABLE III

Acid mucopolysaccharide content of samples of human subcutaneous tissue *

Age	Sex	Total acid mucopolysaccharide			Mucopolysaccharide remaining after hyaluronidase digestion		
		Carbazole	Orcinol	Carbazole/ orcinol	Carbazole	Orcinol	Carbazole/ orcinol
48	M	86.4	111	0.78	52	64	0.82
49	F	106	128	0.83	87	93	0.93
54	M	105	129	0.81	48	70	0.68
65	M	98.9	124	0.80	66	78	0.84
70	F	81.0	144	0.56	62	83	0.74
79	M	145	153	0.95	73	88	0.83
		Average		0.79	Average		0.81

* Figures given are μg. of uronic acid in acid mucopolysaccharide per 100 mg. of dried, fat-free tissue found using carbazole and orcinol determinations. Two hundred mg. samples were used for extraction.

TABLE IV

Acid mucopolysaccharide content of human aorta *

Age	Sex	Total acid mucopolysaccharide			Mucopolysaccharide remaining after hyaluronidase digestion		
		Carbazole	Orcinol	Carbazole orcinol	Carbazole	Orcinol	Carbazole/ orcinol
39	F	792	505	1.6	248	197	1.3
39	M	612	425	1.4	231	178	1.3
41	M	414	283	1.5	199	147	1.4
46	M	573	350	1.6	239	189	1.3
54	M	625	477	1.3	262	224	1.2
		Average		1.5	Average		1.3

* Figures given are μg. of uronic acid in acid mucopolysaccharide per 100 mg. of dried, fat-free tissue found using carbazole and orcinol determinations. Seventy-five mg. samples were used for extraction.

tissues studied was found to be digested by testicular hyaluronidase (Tables II and through V).

Electrophoresis of extracts of the four tissues studied revealed two components which stained metachromatically with toluidine blue. The faster component had the same mobility as control samples of chondroitin sulfate and heparin; these compounds were not separated by this technique. The slower component had the same mobility as control samples of hyaluronate.

In order to determine whether two or three components were present, pyridine and ammonium sulfate were used to precipitate the hyaluronate-like component in each extract, following the method of Jeanloz and Forchielli (9). The pellicle which separated at the interface had the chromatographic characteristics of hyaluronate. The aqueous phase was found to be free of hyaluronate-like material in the propanol system (Table VI, A), but still contained two components in the ethanol system. One of the components remaining in the aqueous phase had the mobility of heparin and the other moved like chondroitin sulfate (Table VI, B).

DISCUSSION

The method described permits a reasonably reliable determination of the quantity of acid mucopolysaccharide present in tissue samples. The levels found are significantly higher than those previously reported for skin (1) and aorta (2). The ratio of hexosamine to uronic acid in the final precipitate obtained from the extracts of all four tissues was usually close to the theoretical ratio of 1.0, establishing that the material found is mucopolysaccharide and of sufficient purity for quantitative studies. Additional evidence of the nature of the material measured included the digestion of a portion of the extract of each tissue by testicular hyaluronidase, the paper chromatographic and electrophoretic mobilities of the compounds present, and their metachromatic staining with toluidine blue.

TABLE V

Acid mucopolysaccharide content of human thyroid tissue *

Diagnosis	Total acid mucopolysaccharide			Mucopolysaccharide remaining after hyaluronidase digestion		
	Carbazole	Orcinol	Carbazole/ orcinol	Carbazole	Orcinol	Carbazole/ orcinol
Normal	127	110	1.2	33	46	0.73
Normal	64.5	93	0.69	20	28	0.73
Colloidal adenoma	151	114	1.3	77	88	0.87
Colloidal adenoma	156	204	0.76	58	70	0.83
Colloidal adenoma	139	173	0.80	75	107	0.70

* Figures given are μg. of uronic acid in acid mucopolysaccharide per 100 mg. of dried, fat-free tissue found using carbazole and orcinol determinations. One hundred fifty mg. samples were used for extraction. Normal thyroids were obtained at autopsy, others by surgical removal.

TABLE VI

Results of paper chromatography of extracts of tissues *

Chondroitin sulfate	Heparin	Hyaluronate	Whole extract	Pyridine pellicle	Aqueous phase
A. Chromatography in buffered propanol					
0.9	0.8	0.1	0.1 and 0.8	0.1	0.8 only
B. Chromatography in buffered ethanol					
0.8	0.1	0.1	0.1 and 0.8		0.1 and 0.8

* Pyridine pellicle refers to the material which separated at the interface when the whole extract in water was treated with pyridine and ammonium sulfate, as described. Aqueous phase refers to the material remaining after removal of the pellicle. Figures given are Rf values at 25° C. for each component observed.

At least one mucopolysaccharide, chondroitin sulfate B, contains iduronic acid. Iduronic acid, compared with glucuronic acid, gives considerably less color in the carbazole determination but slightly more color in the orcinol determination (10). The ratio of carbazole to orcinol values for a sample of chondroitin sulfate B was found to be 0.5, which is similar to previous reports (11). Since glucuronic acid was the standard used in all determinations, the presence of chondroitin sulfate B in tissue extracts in sufficient quantity would produce carbazole to orcinol ratios of less than one. The ratio would be lowered by incubation with hyaluronidase, since material with a higher ratio would be digested leaving the hyaluronidase-resistant chondroitin sulfate B in higher relative concentration. Chondroitin sulfate B has been reported to be the major mucopolysaccharide component of pigskin and bullhide (12). The finding of an average ratio of 0.76, falling to 0.52 after hyaluronidase treatment, indicates the presence of chondroitin sulfate B as a major component of the acid mucopolysaccharide in human dermis. The carbazole to orcinol ratio of 1.5 found in aorta may be due to the presence of compounds such as heparitin sulfate, recently described by Meyer, Hoffman, and Linker, and found to be present in aorta in considerable concentration. This compound was reported to have a carbazole to orcinol ratio around 2.0 (13).

The presence of acid mucopolysaccharide in thyroid tissue has been suggested by its very high hexosamine content (8), histochemical observations (14), and the finding of a fall in the viscosity of colloid following exposure to hyaluronidase (15). Boas and Foley (16) subjected extracts of thyroid tissue to electrophoretic analysis and could account for all the hexosamine on the basis of the proteins present; no uronic acid in mucopolysaccharide could be found by their methods. By our procedure acid mucopolysaccharide was demonstrated to be present in thyroid tissue. Although it contained only one per cent of the total hexosamine, the average concentration of acid mucopolysaccharide was found to be higher than in subcutaneous tissue or dermis.

The chromatographic and electrophoretic characteristic of the three components found cannot be considered to establish their identity. This is particularly notable in the case of heparin, which was used as a standard for reasons of economy; chondroitin sulfate B was found to have the same mobility characteristics in the solvent systems used (4). It is possible that other mucopolysaccharides have characteristics similar to the standards used in this study.

SUMMARY

1. A method is described which permits the determination of the total amount of acid mucopolysaccharide in tissue samples, and gives some information concerning the nature of the mucopolysaccharides present.

2. The total amount of acid mucopolysaccharide present in human dermis, subcutaneous tissue, aorta and thyroid tissue is reported.

3. Electrophoretic and chromatographic studies revealed the presence of metachromatic staining compounds in extracts of each tissue. These were resolved into three components with mobility characteristics resembling control preparations of chondroitin sulfate A, heparin and hyaluronate.

4. Comparison of carbazole and orcinol values for uronic acid indicates the presence of chon-

droitin sulfate B as a major component of the acid mucopolysaccharide in dermis.

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STUDY ON THE EFFECT OF STREPTOKINASE-ACTIVATED PLASMIN (FIBRINOLYSIN) ON CLOTS IN VARIOUS STAGES OF ORGANIZATION [1, 2]

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In previous studies (1, 4), a method was developed for the quantitative recording of the lysis of thrombi and emboli produced with I^{131}-labeled fibrinogen. Using this method, the *in vivo* fibrinolytic activity of human and bovine plasmin was demonstrated (2). Other proteolytic enzymes (crude pancreatic protease, trypsin, carboxypeptidase, papain and ficin) were ineffective in maximal tolerated doses (1, 3, 4). Literature on the therapeutic use of proteolytic enzymes has been reviewed (1, 2). In all previous experiments, treatment was started one-half hour after forming labeled clots in the experimental animals. The question arose whether plasmin treatment would be effective in dissolving organized clots and whether there is a correlation between degree of organization and plasmin-induced lysis. The preparations referred to as "plasmin" in this study actually contain a variety of enzymatic activities; they are fibrinolytic, proteolytic and are able to activate the fibrinolysin system. Presumably they represent a mixture of plasmin, plasmin-activator and free streptokinase. It is difficult to ascribe therapeutic activity to any one of these components. The purpose of these experiments is to study therapeutic indications and conditions for the preparations currently undergoing clinical trial. Labeled clots were produced in several veins of dogs at various time intervals. At the time of treatment, these clots were of various ages. Differences in lysis were established by recording disappearance of radioactivity and by histopathologic study.

MATERIALS AND METHODS

Materials and methods were similar to those described in previous studies (1). Under aseptic conditions, I^{131}-labeled purified bovine fibrinogen was introduced through side branches into isolated segments of veins of dogs and clotted with the aid of thrombin. Semi-constricting aluminum wire ligatures were placed proximal to the clots and artery clamps isolating the segment were released one-half hour after clot formation. Radioactivity over the area was recorded with the aid of a specially constructed lead shield, a scintillation counter, an analytical radiation rate meter, and an Esterline recorder.

Mongrel dogs, 10 to 16 Kg. in body weight, were used. At the time of the beginning of treatment, the clots varied in age from 30 minutes to 10 days. In most dogs two clots of the same age were present; one was removed before institution of plasmin treatment. All other clots were removed after the last treatment. Sections from at least two areas of each clot were examined histologically.

Five daily intravenous doses of human plasmin, 30 plasmin units (Loomis) per Kg.,[3] were given. One mg. of the plasmin preparation used was found to contain 3 fibrinolytic plasmin units (Loomis) or 0.098 proteolytic casein unit (6). The specific activity was 0.28 casein unit per mg. protein. This dose was previously found (2) to cause lysis of fresh clots but not to alter fibrinogen and prothrombin levels. In most previous studies the dose was administered in four hour infusions; in the present experiments, rapid intravenous injections were given. In the previous studies, human plasmin was prepared from Cohn's Fraction III according to the method of Kline (7). In the present study a simple streptokinase-activated acid extract of Fraction III was used, prepared by Dr. Werner Baumgarten, Sharp and Dohme Division of Merck and Co., West Point, Pa. The following information was made available on the method of preparation of this material. Fraction III, prepared by the

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[3] A plasmin unit (Loomis) is defined as the quantity of enzyme needed to dissolve 0.6 ml. of a 0.3 per cent purified bovine fibrin clot at 45° C. in two minutes at pH 7.2 (5).

alcohol fractionation method of Cohn, was extracted with acid, adjusted to pH 7.8, and to each equivalent of 40 mg. of Fraction III, 13,000 Christensen units of streptokinase was added (U. S. Patent 2,666,729). We found that when lyophilized samples of the above material were diluted with increasing volumes of physiological saline, fibrinolytic activity first increased, then decreased. Such bell-shaped dilution versus activity curves could be duplicated by adding excessive amounts of streptokinase to plasminogen prepared by the Kline method. This was interpreted as indicating that the preparation used in this study contained a considerable amount of free streptokinase. It is known that most batches of streptokinase are poor activators of dog plasminogen. On the other hand Sherry, Titchener, Gottesman, Wasserman, and Troll have shown (8) that streptokinase-activated human plasmin preparations may activate dog plasminogen. It is presently a matter of controversy whether this effect of streptokinase-activated human plasmin is due to the presence of "activator" (9), or to the activating ability of a hypothetical plasminogen-streptokinase complex (10, 11), or a plasmin-streptokinase complex (4, 11).

Blood samples were taken before, and at various time intervals after, plasmin injections for hematological and biochemical analyses as in previous studies (1–4). Prothrombin time is reported as clotting index: the ratio of the prothrombin time prior to treatment to the prothrombin time during and after plasmin administration. In most experiments as in previous studies (1–4) prothrombin time was also determined after adding bovine fibrinogen, so as to eliminate the effect of fibrinogenopenia on prothrombin time determinations.

RESULTS

Clot lysis

Figure 1 shows an experiment in which a dog with one-half hour, one, two and three day old clots was treated with daily injections of 30 plasmin units (Loomis) per Kg. of human plasmin. The first point on the graph indicates radioactivity over the thrombus at the time of clot formation; the second represents radioactivity immediately

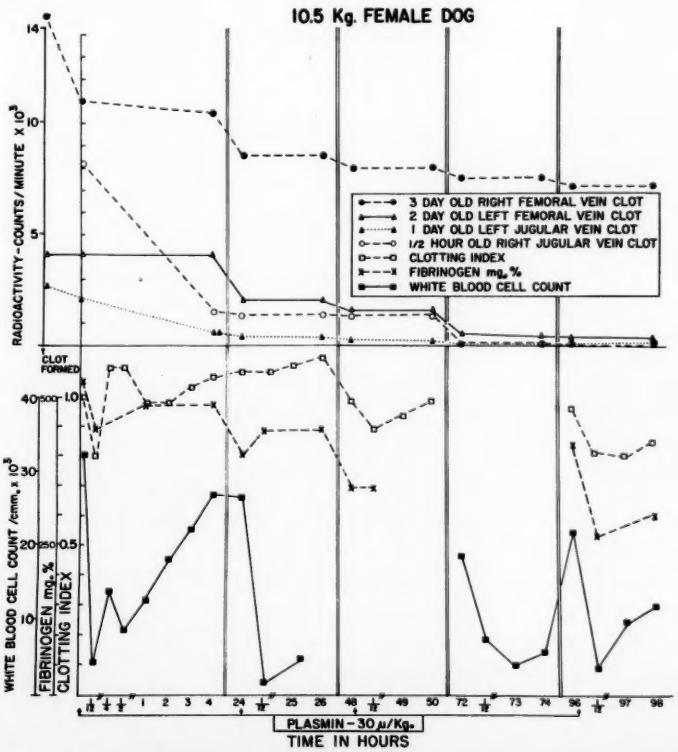
10.5 Kg. FEMALE DOG
RADIOACTIVITY - COUNTS / MINUTE X 10³
3 DAY OLD RIGHT FEMORAL VEIN CLOT
2 DAY OLD LEFT FEMORAL VEIN CLOT
1 DAY OLD LEFT JUGULAR VEIN CLOT
1/2 HOUR OLD RIGHT JUGULAR VEIN CLOT
CLOTTING INDEX
FIBRINOGEN mg.%
WHITE BLOOD CELL COUNT
CLOT FORMED
WHITE BLOOD CELL COUNT /cmm. x 10³
FIBRINOGEN mg.%
CLOTTING INDEX
PLASMIN - 30 µ/Kg.
TIME IN HOURS

FIG. 1. EFFECT OF PLASMIN ON ONE-HALF HOUR, ONE, TWO AND THREE DAY OLD CLOTS, CLOTTING INDEX, FIBRINOGEN LEVEL AND WHITE BLOOD CELL COUNT

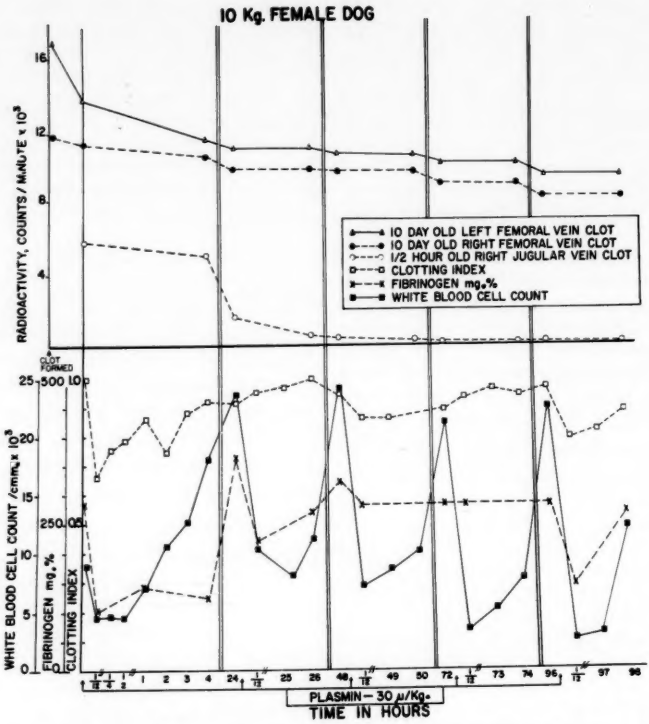
10 Kg. FEMALE DOG
RADIOACTIVITY, COUNTS / MINUTE x 10³
10 DAY OLD LEFT FEMORAL VEIN CLOT
10 DAY OLD RIGHT FEMORAL VEIN CLOT
1/2 HOUR OLD RIGHT JUGULAR VEIN CLOT
CLOTTING INDEX
FIBRINOGEN mg.%
WHITE BLOOD CELL COUNT
CLOT FORMED
WHITE BLOOD CELL COUNT /cmm. x 10³
FIBRINOGEN mg.%
CLOTTING INDEX
PLASMIN – 30 μ/Kg.
TIME IN HOURS

FIG. 2. EFFECT OF PLASMIN ON ONE-HALF HOUR AND 10 DAY OLD CLOTS, CLOTTING INDEX, FIBRINOGEN LEVEL AND WHITE BLOOD CELL COUNT

before initiation of treatment. It can be seen that I^{131}-labeled clots up to the age of two days completely disappeared during the five day treatment period. Only slight lysis was evidenced in the three day clot. It should be remembered, however, that statistical analysis of lysis of all three day old clots versus control clots revealed no significant difference. Figure 2 illustrates an experiment in which a dog with two 10 day old clots and one-half hour old clot was treated. Radioactivity over the half-hour old clot disappeared as in the previous experiment, but the 10 day old clots showed no significant lysis compared to clots in untreated dogs (2). Results with five day old clots were identical with those of 10 day old clots. Fresh I^{131}-labeled fibrinogen was prepared before each series of experiments. Thus, clots of various ages produced in the same dog had various starting levels of radioactivity. No relationship was found between base radioactivity levels and degree of lysis.

TABLE I

Effect of clot age on lysis

Age of clots	No. of clots	Treatment of dogs	Mean of % lysis and standard error of mean at end of treatment period
½ hr.	23	saline (control)	23.54 ± 3.17
10 days	4*	30 plasmin units (Loomis) per Kg. body weight	23.72 ± 3.75
5 days	4		22.43 ± 2.63
3 days	10		20.49 ± 3.55
2 days	6		80.17 ± 4.26
1 day	6		80.53 ± 4.79
½ hr.	6†		97.42 ± 1.10

* Eight additional clots gave similar results but are not included since somewhat different methods were used.

† Three additional clots gave similar results after treatment with more purified plasmin preparations.

Table I summarizes the results of all clots investigated in this study. It appears that under the conditions of these experiments and at the dose

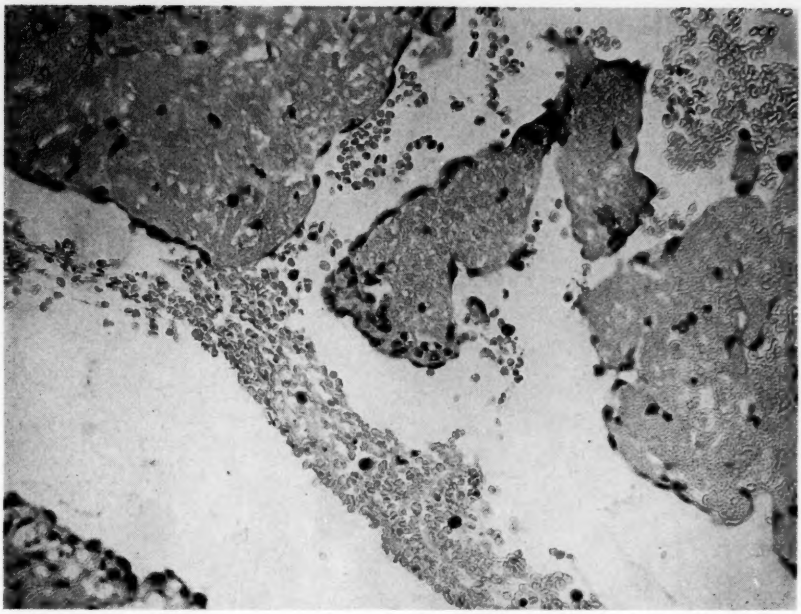

FIG. 3. THREE DAY OLD CLOT WITH ENDOTHELIAL COVERING OVER THE ENDS (HEMATOXYLIN EOSIN 420 ×)

level used, clots three days of age or older can not effectively be dissolved with human plasmin.

Histopathologic findings

The histological findings were not uniform throughout the length of a clot. Despite this it was possible to grade the clots histologically and it was found that the histological picture correlated closely with their known age. The most important factor in grading was the degree of endothelial and fibroblastic activity and subsequent vascularization and fibrosis. The ends of the clot and spaces where the clot did not adhere firmly to the vessel wall were largely covered with endothelium by the third day (Figure 3) and about this time a few fibroblasts were occasionally present in the substance of the clot (Figure 4). Vascular spaces were formed soon after this (Figure 5). By the tenth day all clots were fully vascularized and many were only partially attached to the vessel wall with blood flowing around them (Figure 6). A second factor in grading was the change in the clot itself from the early mixture of fibrin and red cells to a more homogeneous mass from which the red cells gradually disappeared. Van Giesen stain revealed no collagen formation before the tenth day.

It was not possible to distinguish the clots which had received plasmin therapy after three or more days of existence from the control clots of similar age. Where treatment was given earlier, the clot appeared to be less advanced than expected at the time of removal. In some vessels where complete disappearance of the original isotopically-labeled fibrin structure was indicated by measurement of radioactivity, histologic examination revealed the presence of relatively fresh nonlabeled clots. It seemed probable that lysis of the original clot had been followed by formation of a fresh thrombus. Control clots of the same age showed little decrease of radioactivity levels. A varying degree of inflammatory reaction was always present in the vessel wall as a result of the manipulations necessary in the daily measurement of radioactivity. This may have influenced the reformation of the clot after the initial lysis. Occasionally, organization appeared less advanced than expected in the presence of severe necrosis of the vessel wall.

FIG. 4. FOUR DAY OLD THROMBUS WITH FIBROBLASTS IN SUBSTANCE OF CLOT (HEMATOXYLIN EOSIN 420×)

Biochemical findings

In previous experiments (1, 2), human plasmin, prepared according to the method of Kline and activated with streptokinase in a dose of 30 plasmin units (Loomis) per Kg. (4) had no effect on the fibrinogen and prothrombin level if given as a four hour intravenous infusion. In this study, acid extracts of Cohn's Fraction III activated with streptokinase were given by rapid intravenous injection. This resulted in a fall of fibrinogen levels as well as clotting index after the first injection. These changes did not usually occur during subsequent injections, but occasionally reappeared at the fifth daily injection.

Hematologic findings

Leukopenia was observed after each injection. No decrease of this change occurred during prolonged daily treatment. Leukopenia does not appear to be due to the enzymatic effects of plasmin since plasminogen was shown to cause the same changes (2), and since Guest, Murphy, Bodnar, Ware, and Seegers (12) produced similar effects with inactivated bovine plasmin. Red blood cell counts, hematocrit and hemoglobin fluctuated within normal daily limits.

Blood pressure

In many dogs, carotid blood pressure was recorded during and after administration of plasmin. Rapid infusions of 30 plasmin units (Loomis) per Kg. produced a small decrease of blood pressure (7 to 10 mm. Hg) which returned to normal within 10 minutes. In previous studies (2), a gradual decrease in the hypotensive activity was noticed during daily treatment with plasmin.

DISCUSSION

It appears from this and previous studies (2) that during daily human plasmin treatment, resistance gradually develops toward its fibrinolytic, hypotensive, fibrinogenopenic and prothrombinopenic, but not its leukopenic effects.

Resistance to lysis is apparent from the observation that most of the fibrinolytic effect is obtained within 24 hours after the first injection of plasmin, the remaining portions of the clot exhibiting less and less lysis during the subsequent days of treatment (2). It was found that the ability of plasmin at a dose level of 30 plasmin units (Loomis) per Kg. to dissolve a clot is closely correlated with the degree of organization of the clot; the older the clot, the less lysis. It may be significant that even by the third day, most of the free edges of the clot were covered with a layer of endothelial cells (see Figure 3). No extensive histochemical study was made of the changes in the fibrin mass but collagen was not demonstrated before the tenth day. Williams (13), in a detailed study of the organization of artificially produced arterial clots, noted rapid endothelialization, occurring in the first 48 hours. He demonstrated a change in the staining reaction accompanying condensation of the fibrin after four days. Collagen appeared in the second week. It seems probable that the presence of endothelial cells may act as a physical barrier between the plasmin and the

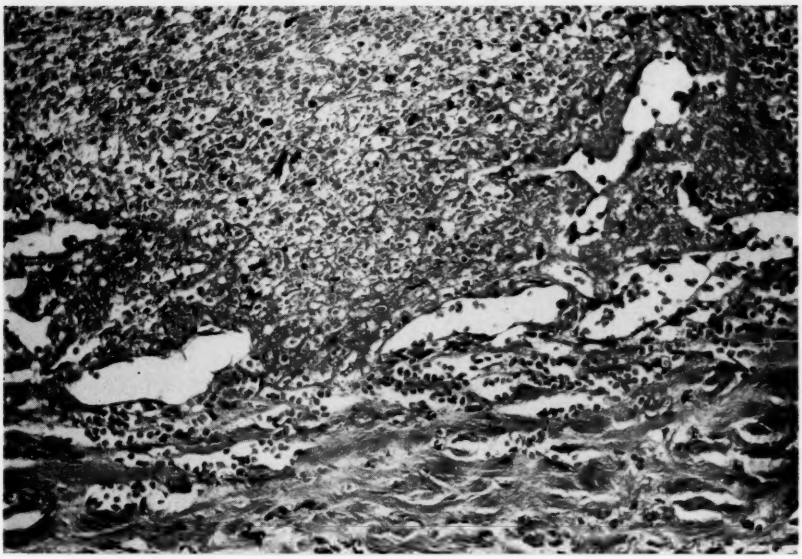

FIG. 5. FIVE DAY OLD CLOT, SHOWING VASCULARIZATION (HEMATOXYLIN EOSIN 420 ×)

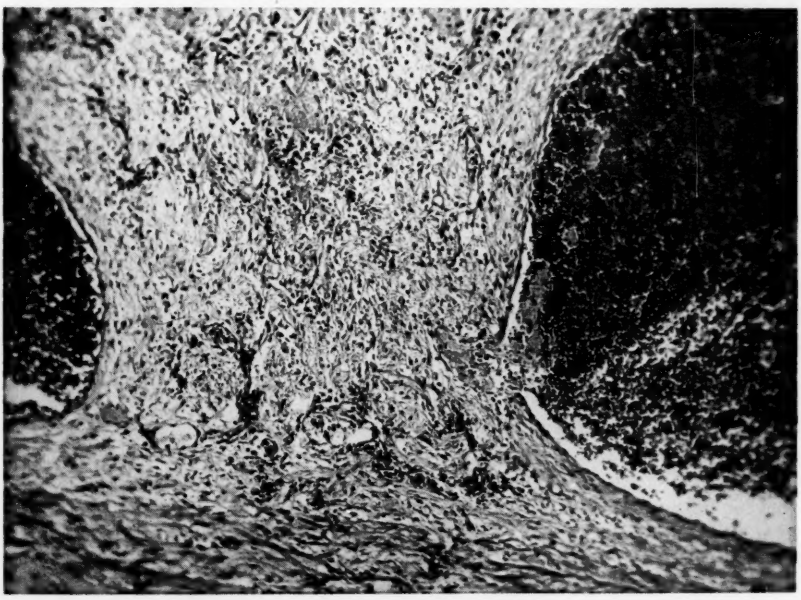

FIG. 6. TEN DAY OLD CONTROL CLOT WITH EXTENSIVE VASCULARIZATION AND PARTIAL DETACHMENT FROM THE VESSEL WALL (HEMATOXYLIN EOSIN 200 ×)

Note fresh blood flowing through vessel between clot and wall.

fibrin clot. In addition, increased density of the fibrin may also impair the effect of plasmin.

The above factors may explain resistance to lysis, but are certainly inadequate to account for the resistance toward biochemical and hypotensive changes. A study of changes in antiplasmin levels and antiplasmin antibody formation is presently under way. Results to date indicate that although chronic treatment with plasmin will induce fluctuations in these factors, they are insufficient to explain the degree of resistance encountered.

From the therapeutic point of view, it appears that acute thrombo-embolic disorders present better indications for plasmin therapy than chronic conditions. Payling-Wright (14) has suggested the possibility that cortisone given simultaneously with anticoagulants may delay organization of clots and increase the rate of recanalization of thrombosed vessels in rabbits. On the other hand, there are indications that cortisone may increase antiplasmin production or activity thus decreasing normal fibrinolytic activity of the blood (15).

After successful lysis of acute clots, reformation was observed in spite of continued treatment with plasmin. The experimental procedure called for opening the wound daily and inserting a lead shield and scintillation counter in the area. This obviously resulted in some degree of trauma and represented a possible source of infection despite daily administration of antibiotics. In addition, inflammatory reactions might have contributed to thrombus formation. These new clots were nonradioactive and were detected only upon autopsy. The fact that they did not lyse during daily treatment with plasmin (although they were fresh, unorganized clots) may be an expression of the general resistance developed toward plasmin as discussed above. Under most clinical conditions, stimuli for clot reformation are undoubtedly less than in the experimental arrangement reported here. The possibility of clot reformation after successful fibrinolytic therapy should be considered, particularly if a) the conditions originally responsible for clot formation still persist, and b) the temporary presence of the clot caused additional injury to the vessel wall.

CONCLUSIONS

Venous clots were produced with I^{131}-labeled fibrinogen in dogs at various time intervals. Five daily treatments with 30 plasmin units (Loomis) per Kg. of human plasmin completely lysed clots less than three days old, but did not affect those older than three days. Histologic examination revealed that three days after formation, the clot is already largely separated from the blood stream by a layer of endothelial cells which may prevent plasmin from reaching the clot. Increased clot density may also be a factor in the clot becoming resistant to treatment.

In some instances, although the original radioactive clot completely disappeared, reformation of new, nonradioactive thrombi took place. Trauma might have been a major cause of this phenomenon. Reformation of the clot occurred in spite of daily administrations of plasmin. This may be related to the developing resistance of the animal to this agent as shown by decrease in changes of the fibrinogen levels, clotting index and blood pressure response (2, 4, 16).

On the basis of these findings, it seems that better therapeutic results may be expected from intravenous treatment with plasmin in acute rather than chronic thromboembolic disorders. In the latter, results would appear to depend on the degree of organization of the clot.

ACKNOWLEDGMENT

This study was suggested by Dr. George E. Moore, Director, Roswell Park Memorial Institute, for whose advice the authors wish to express their gratitude. The assistance of J. W. Byron and Donna Halloran is gratefully acknowledged.

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PROCEEDINGS OF THE FIFTIETH ANNUAL MEETING OF THE AMERICAN SOCIETY FOR CLINICAL INVESTIGATION HELD IN ATLANTIC CITY, N. J., MAY 5, 1958

PRESIDENTIAL ADDRESS

CLINICAL INVESTIGATION AND MEDICAL EDUCATION

BY RICHARD V. EBERT

I have chosen as the subject of my address the contribution of clinical investigation to medical education. During the past few years there has been an extensive re-evaluation of the method and content of medical teaching. The discussion that has ensued cannot but be helpful in defining the goals of our medical schools.

It would seem presumptuous of me to emphasize the importance of the relationship of investigation to teaching if this aspect had not been relatively neglected in recent writings on medical education. Whitehead (1) has stated, "The justification for a University is that it preserves the connection between knowledge and the zest of life, by uniting the young and old in the imaginative consideration of learning. . . . This atmosphere of excitement, arising from imaginative consideration transforms knowledge. A fact is no longer a bare fact: It is invested with all its possibilities. It is no longer a burden on the memory: It is energising as the poet of our dreams, and as the architect of our purposes." The clinical investigator can bring to the student an imaginative consideration of learning. This can transform the discussion of clinical problems on the ward or in the conference room from the dull repetition of clinical syndromes or exposition of traditional therapy to the excitement which can be evoked by exploring the frontiers of knowledge. My own life was profoundly influenced by two great teachers, Soma Weiss and William Castle. They brought the freshness and enthusiasm of the investigator to each clinical problem. The student was not only stimulated to acquire the known information on a subject but was also compelled to explore the unknown.

The present day criticism of medical education does not lie in the failure of the teachers to transmit information in an imaginative and enthusiastic manner. Rather it pertains to the neglect of certain areas of human knowledge which are relevant to the care of patients and to a failure to teach the student to understand the patient as a human being. There is considerable truth in the first indictment. One of the major failings of modern science is the division of knowledge into compartments. Most of us have difficulty in mastering the techniques and new contributions in our own narrow field. For this reason it is easy to overlook major advances in other areas of knowledge. Certainly the increasing knowledge of man in relationship to society should contribute greatly to medicine. We should welcome the contribution of the social scientist, the anthropologist or the psychologist to the solution of the problems of the sick human being.

Admitting the need for a greater understanding of man and his relationship to society, is there any need to abandon the scientific method or alter our critical standards? I believe not. We must guard against the confusion of hypothesis with fact and poor logic in this area as in any other. As Romano (2) has put it in discussing the teaching of psychiatry, we need more, not less, science in medicine.

If we accept a broad definition of science there need be no other aspect of the teaching of medicine than the scientific. Morris Cohen (3) has said, "Scientific method is thus the persistent application of logic as the common feature of all reasoned knowledge. From this point of view scientific method is simply the way in which we test impressions, opinions or surmises by examining the best available evidence for and against them." If this be true then every physician should have proper training in the use of the scientific method. Certainly the heart of the practice of medicine is the critical evaluation of evidence and the ability to select a logical course of action based on this evaluation.

Experience in scientific research would appear to be the best method of inculcating the use of the scientific method into teacher and student. It is particularly important for the teacher of medicine. It is only too easy in clinical teaching to substitute authority for knowledge and prejudice for sound reasoning. This does not imply that the teacher need be a leader in one of the fields of research or even be continuously active in research.

An important development is the increasing opportunity for the undergraduate to participate in research. This is being done by creating more free time in the academic year and by providing fellowships for summer work. Probably nothing is more valuable to the student than the opportunity to participate in scientific investigation. Only in this way can he be made to understand the difficulty in evaluating evidence obtained from experimentation. The rapid growth of scientific knowledge in medicine and the accelerated introduction of new therapeutic agents by the pharmaceutical companies strains the critical faculties of every physician. Nothing is more important to the medical student than to acquire the ability to accurately evaluate evidence. In the past the rigid curriculum together with the necessity of memorizing

large amounts of information tended to stifle the imagination of the student. Thus his most precious asset was lost.

One of the encouraging aspects of recent changes in medical education has been the breakdown of departmental barriers. The science of medicine must have a wholeness which is more than the sum of the individual disciplines which are represented by the various departments in a medical school. The necessity for a high degree of specialization in research is apparent. The acquisition of difficult techniques and a thorough knowledge of the mass of information which has been accumulated in a narrow area is essential for the investigator. In teaching, a broad approach is important. The medical student must learn to understand man in health and disease.

Perhaps what I have said seems familiar and of no pertinence to the present problems of medical education. Certainly all of these views were expressed by Flexner (4). Yet in some of the recent discussions of medical education there are implications that the intuitive approach to medical problems has value in teaching. As Atchley (5) has pointed out, there have also been implications that training in and adherence to the scientific method in some manner diminishes the power of compassionate feeling towards suffering human beings.

It is my feeling that the teaching of medicine must rest on the scientific method. The scope of inquiry and interest cannot be confined to the purely biologic or physical aspect of the problems presented by the sick human being but must include the psychological and social areas. In broadening the area of inquiry we should not lose our critical sense. In medicine there is always the temptation to accept new ideas with enthusiasm and without careful examination. This must be guarded against in the evaluation of contributions of the social sciences to medicine. Equally dangerous, however, is the closed mind which limits the boundaries of science to the purely physical and chemical aspects of nature. One of the great challenges to medicine in our time is a better understanding of man's relationship to man. Every encouragement should be given to the scientific workers in this difficult field.

The ferment in medical education is a sign of the vitality of American medicine. The numerous experiments in teaching should provide information of great value. Out of this may grow a diversity in the approach to medical education which has been lacking in the past. I would certainly hope that no attempt will be made to impose a uniform philosophy of medical education. Such a philosophy would eventually stultify the thinking of the teachers and create an atmosphere of conformity. Instead, an atmosphere of freedom and experimentation should exist in medical education as in scientific research.

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PAPERS PRESENTED AT THE FIFTIETH ANNUAL MEETING 1958

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2. The Effect of Parathyroid Extract (PTE) on the Renal Clearance of Diffusible Calcium. CHARLES R. KLEEMAN, ROBERT E. ROCKNEY, and MORTON H. MAXWELL, Los Angeles, Calif. (907)

3. Effects of Reduced Glomerular Filtration on Urine Concentration in the Presence of Antidiuretic Hormone. NORMAN G. LEVINSKY, DOUGLAS G. DAVIDSON, and ROBERT W. BERLINER, Bethesda, Md. (910)

4. Reappraisal of Renal Hemoglobin Excretion: The Differential Transport of Free and Protein-Bound Hemoglobin. WILLOUGHBY LATHEM, Pittsburgh, Pa. (909)

5. Adequacy of Renal Oxygenation in Essential Hypertension. WILLIAM E. HUCKABEE, Boston, Mass. (903)

6. Physiological Diagnosis of Disordered Coronary Circulation. RICHARD GORLIN, NORMAN BRACHFELD, PIERRE BOPP, and COLIN MACLEOD, Boston, Mass. (898)

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8. The Pulmonary Collateral Blood Flow in Man. ALFRED P. FISHMAN, GERARD M. TURINO, MARTIN BRANDFONBRENER, AND AARON HIMMELSTEIN, New York, N. Y. (894)

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11. Platelet Thromboplastic Factor: Its Chemical Nature, *In Vitro* Activity, and the Identification of Similar Thromboplastic Substances in Red Blood Cells. STANLEY B. TROUP and CLAUDE F. REED, Rochester, N. Y. (937)

12. An Investigation of Purine and Pyrimidine Excretion in Normal and Leukemic Subjects Utilizing Ion Exchange Column and Paper Chromatographic Techniques. WILLIAM S. ADAMS, WILLIAM A. SKOOG, and FRANCES W. DAVIS, Los Angeles, Calif. (875)

13. Dynamics of Proliferating Cell Systems of Man Studied with Tritiated Thymidine. EUGENE P. CRONKITE, THEODOR M. FLIEDNER, JOSEPH R. RUBINI, VICTOR P. BOND, and WALTER L. HUGHES, Upton, New York. (887)

14. The Effect of Intravenously Administered Ceruloplasmin on Copper Absorption in a Patient with Wilson's Disease. IRMIN STERNLIEB, ANATOL G. MORELL, and I. HERBERT SCHEINBERG, New York, N. Y. (934)

15. The Mechanism of the Protein-Sparing Effect of Glucose. JEAN D. WILSON and MARVIN D. SIPERSTEIN, Dallas, Texas. (943)

16. Stimulation of Amino Acid Penetration into Mammalian Skeletal Muscle by Insulin. DAVID M. KIPNIS and MATTHEW M. NOALL, St. Louis, Mo. (906)

17. Modification of Adrenal Steroid Patterns in Man by Means of an Inhibitor of 11β-Hydroxylase. GRANT W. LIDDLE, DONALD ISLAND, HERSCHEL ESTEP, and GORDON M. TOMKINS, Nashville, Tenn., and Bethesda, Md. (912)

18. Steroid Metabolism in the "Salt-losing" Form of Congenital Adrenal Hyperplasia. WALTER R. EBERLEIN and ALFRED M. BONGIOVANNI, Philadelphia, Pa. (889)

19. The Influence of ACTH on the Release of Non-Esterified Fatty Acids from Rat Adipose Tissue *In Vitro*. J. EARLE WHITE and FRANK L. ENGEL, Durham, N. C. (942)

20. Effect of Growth Hormone on Plasma Fatty Acids. M. S. RABEN and C. H. HOLLENBERG, Boston, Mass. (922)

21. The Measurement of Serum Sulfation Factor as an Index of Somatotropin Activity in Man. WILLIAM H. DAUGHADAY, WILLIAM D. SALMON, JR., and FRANCE ALEXANDER, St. Louis, Mo. (888)

22. Mechanisms of Body Fluid Enzyme Alterations in the Absence of Tissue Necrosis. FELIX WRÓBLEWSKI, ALICE E. MOORE, CARLOS MANSO, and KANEMATSU SUGUIRA, New York, N. Y. (943)

23. Chemical Constituents of Pine Pollen and Their Possible Relationship to Sarcoidosis. MARTIN M. CUMMINGS and PAUL C. HUDGINS, Washington, D. C. (888)

24. The Mechanism of Tolerance to Fever. ABRAHAM I. BRAUDE, MARGARET ZALESKY, and HERNDON DOUGLAS, Pittsburgh, Pa. (880)

25. Anergy and the Response to Homografts in Hodgkin's Disease. W. D. KELLY, R. A. GOOD, and R. L. VARCO, Minneapolis, Minn. (906)

26. A Circulating Antibody Directed Against Penicillin. ALLYN B. LEY, AMOS CAHAN, and KLAUS MAYER, New York, N. Y. (911)

27. Intestinal Similarities Between Celiac Disease and Idiopathic Sprue. CYRUS E. RUBIN, LLOYD L. BRANDBORG, PATRICIA C. PHELPS, and HAWLEY C. TAYLOR, JR., Seattle, Wash. (927)

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ABSTRACTS

An Investigation of Purine and Pyrimidine Excretion in Normal and Leukemic Subjects Utilizing Ion Exchange Column and Paper Chromatographic Techniques. WILLIAM S. ADAMS, WILLIAM A. SKOOG, and FRANCES W. DAVIS, Los Angeles, Calif. (Introduced by William N. Valentine).

Although extensive studies of uric acid excretion in leukemic subjects have been reported in the past, very little is known of the urinary excretion of other purines and pyrimidines in this disease. Earlier workers have been hampered by the lack of suitable methods for investigating these substances. Our laboratory has developed a method which consists of the absorption of an aliquot of a 24 hour urine on a Dowex 2 anion exchange column and fractional collection of the effluent using a pH gradient elution technique. The elution properties, R_f values obtained from conventional two phase paper chromatographic methods, and ultraviolet absorption spectra have been compared with known standards. This method permits the separation, identification and quantitation of products of nucleoprotein degradation in urine.

The elution patterns from 4 normal and 40 leukemic patients have been studied. Results indicate that normal urine contains cytosine, uracil, uridine, guanine, inosine, adenine and uric acid. Marked quantitative differences in the excretion of some of these substances have been noted in all leukemic subjects studied to date. Moreover, cytidylic acid, thymine and guanosine have been detected only in leukemic urine. Increased inosine excretion has been found in the urine of patients with acute leukemia and chronic lymphocytic leukemia, whereas guanosine has only been found in urine of patients with chronic granulocytic leukemia.

Insufficient data are as yet available to enable one to relate a pattern of excretion to a particular morphological type of leukemia. However, it is possible that the results of these investigations will permit a more accurate differentiation of the acute leukemias than is presently possible on the basis of existing morphologic or tissue culture techniques. Furthermore, if specific biochemical abnormalities are demonstrated, it may be possible to design more suitable antimetabolite therapy in the treatment of these diseases.

The Effect of Anesthetic Agents on Water Diuresis. ARTO H. APRAHAMIAN, JAMES L. VANDERVEEN, JOHN P. BUNKER, ANNE P. MURPHY, and JOHN D. CRAWFORD,* Boston, Mass.

The commonly observed failure of intra- and postoperative patients to develop water diuresis in circumstances which would normally so predispose and where such a response would be of homeostatic value has prompted an investigation of the role of anesthetic agents in diuresis inhibition. Nineteen young adult female patients scheduled for elective surgery were studied. All were in good health. On arrival in the operating suite, fasting but not premedicated, a brisk diuresis was induced by rapid intravenous infusion of 5 per cent dextrose and water. There was no delay in the development of diuresis ascribable to apprehension or other factors. Atropine was then given without effect on the diuresis and finally anesthesia was induced using ether, nitrous oxide, cyclopropane or thiopental. Measurements were made of urine flow, urine and plasma osmolality and of the arterial blood levels of the anesthetic agents.

Induction with ether, nitrous oxide or cyclopropane regularly caused abrupt and long lasting inhibition of the established water diuresis. The responses were similar but not strictly comparable to the inhibition resulting from intravenous vasopressin. Induction with thiopental was without effect on the diuresis. Furthermore, patients anesthetized with thiopental continued diuresis during the subsequent addition of any one of the three inhalation agents. During thiopental anesthesia water diuresis could be inhibited by exogenous intravenous vasopressin and was noted to subside spontaneously when water loading was terminated.

It is concluded that thiopental has no antidiuretic effect of its own and, at anesthetic levels, it prevents the antidiuretic response to ether, nitrous oxide and cyclopropane. These data suggest the possibility that use of thiopental may serve to avoid for the surgical patient the intra- and postoperative period of high susceptibility to water intoxication which is due, at least in part, to the prolonged fixed antidiuresis caused by certain of the commonly used inhalation agents.

Studies of Glucuronide Synthesis and of Glucuronyl Transferase Activity in Liver and Serum. IRWIN M. ARIAS, BERTRAM A. LOWY, and IRVING M. LONDON,* New York, N. Y.

In studies on the mechanism of conjugation of glucuronic acid with bilirubin and other receptors, evidence has been obtained which is suggestive of a new pathway in glucuronide formation. On incubation of 4-methyl umbelliferone (4-MU) with *a*) glucuronic acid (GA), *b*) adenosine triphosphate (ATP), *c*) uridine triphosphate (UTP), and *d*) the soluble fraction of rat liver homogenate, 4-MU glucuronide is formed. Preliminary studies indicate that alpha-glucuronic acid-1-phosphate (GA-1-P) can substitute for GA and ATP. In this system, glucuronide synthesis occurs primarily with the soluble fraction of liver, whereas the formation of glucuronides from uridine diphosphate glucuronic acid (UDPGA) involves glucuronyl transferase which is present in microsomes. This capacity of liver to synthesize glucuronides from glucuronic acid is pertinent to the observation of Danoff and Holt that the administration of GA to

* Member.

infants with erythroblastosis or physiologic jaundice results in reduction of the serum concentration of indirect-reacting bilirubin, presumably by promoting the formation of bilirubin glucuronide.

Previous studies have established that glucuronyl transferase, which is present in liver microsomes of man and several animal species, transfers GA from UDPGA to various receptors including bilirubin, and have shown a specific deficiency of this enzyme in Gilbert's disease. To investigate further the role of this enzyme in this and other clinical states, a highly sensative assay which employs 4-MU, a fluorescent compound, as the receptor has been developed and applied to liver obtained on needle biopsy and to serum. Marked diminution in glucuronyl transferase activity (GTA) was observed in liver biopsy specimens of six patients with nonhemolytic acholuric jaundice of varying severity (serum bilirubin, 1.8 to 22 mg. per cent). In the serum of normal rats, rabbits, dogs or humans, GTA was not detected. But in animals with hepatic necrosis produced by administration of carbon tetrachloride, in three patients with massive hepatic necrosis and in five of seven patients with viral hepatitis, GTA was demonstrable in the serum. To effect glucuronide synthesis in serum, UDPGA was required.

Quantitative Variations in the Nucleohistone Binding Properties of Human Serum. JOHN ARNOLD and CHARLES PAK, Chicago, Ill. (Introduced by Richard L. Landau).

The serum of patients with systemic lupus erythematosus (SLE) has been shown to contain factor(s) with a special affinity for nucleohistone (NH). These factors are difficult to estimate quantitatively and probably escape detection entirely when they occur in low concentrations. The customary determination of these factors by the lupus erythematosus cell test depends in part on the process of phagocytosis. A new quantitative and sensitive method for measuring the affinity of serum components to NH was devised to explore the variations of this property of serum in SLE and in the serum of patients with other dysproteinemias. This method is based on the ability of a nucleohistone film at the air-water interface to react to a test substance by changes in the surface area of the NH film when serum components either bind or penetrate the film.

Under prescribed conditions of pH, temperature, and ionic strength of substrate solution, source and state of polymerization of the NH film and lateral pressure on the film, this method will reveal the presence of factors with an affinity for NH when the serum concentration is as low as one part serum per million parts of substrate solution. The method gives results reproducible to within ± 2 per cent.

The serum of 15 patients with histologically verified SLE was compared to the serum of normal people and to the serum from patients with a variety of plasma protein abnormalities. The nucleohistone films gave a several-fold greater increase in area than did the sera from other sources. However, under suitable conditions the sera from all sources tested gave evidence of a limited degree of NH binding.

By using different sources of NH it has been possible to show that the component of human serum responsible for the NH binding phenomenon is not a single molecular species.

Leukokinetics as Measured with Radioactive Diisopropylfluorophosphate (DFP^{32}). JOHN W. ATHENS, ALVIN M. MAUER, HELEN ASHENBRUCKER, and GEORGE E. CARTWRIGHT,* Salt Lake City, Utah.

Previous work in this laboratory has demonstrated that leukocytes can be labeled with diisopropylfluorophosphate containing radioactive phosphate (DFP^{32}).

Two mg. of DFP^{32} containing 100 to 200 μc. per mg. of DFP is injected intravenously. The leukocytes are isolated from 20 ml. of blood by dextran sedimentation of the red cells followed by gramicidin-lysolecithin hemolysis of the red cells remaining in the supernatant suspension. Platelets are removed by differential centrifugation. The resulting leukocyte sample is plated between two scintillating plastic squares and the radioactivity is determined in a scintillation counter.

The shape of the radioactivity curve obtained in 50 normal subjects has been consistent and reproducible. The curve consists of three phases: a rapid exponential decrease in the first 48 hours after injection of the label (Phase 1); a second period of almost constant radioactivity (Phase 2); and a final exponential decrease in activity (Phase 3). The mean $T_{1/2}$ (± 1 S.D.) of Phase 1 was 5.8 ± 2.2 hours. Phase 2 terminated 11.5 days after labeling (range, 9 to 16 days). The $T_{1/2}$ of Phase 3 averaged 3 days (range, 2 to 6 days).

No evidence has been obtained that any one of the three phases can be explained by reutilization of the label, by elution of the label, or by removal of cells damaged by the label. Since most, if not all, of the leukocyte radioactivity is present in granulocytes, this method provides a means for studying the steady state kinetics of granulocytes. Several possible models for granulocyte kinetics will be presented and discussed.

Studies of the Properdin System in Normal Humans, in Infections and in Malignant Blood Dyscrasias, Using the Phage Technique for Its Measurement. ALDONA BALTCH and PAUL A. BUNN,* Syracuse, N. Y.

Properdin is a naturally occurring protein constituent of human serum. Its concentration varies under certain abnormal conditions of health, *e.g.*, in acute infections and in some blood dyscrasias. Its concentration in serum is measured by determining the degree of its virucidal activity against *E. coli* phage T_5r+. Serum from 30 normal adult subjects has been tested. In the majority (20 subjects) levels between 6 and 16 are found.

Serial determinations of levels of properdin have been made in 90 patients with acute and chronic infections. Serum from staphylococcal infections (20 with positive blood cultures, 12 with serious local infections) revealed

that early in the course of the disease, concentrations fell to below four units. Within four to six days levels rose to normal in those who recovered. Concentrations of properdin remained below two units in those who died. Similar experiences were recorded in acute infections caused by group A streptococci, in Gram-negative bacterial sepsis, in pneumococcal and mixed pneumonia, and in acute lung abscess and empyema. Patients with subacute bacterial endocarditis, Asian influenzal infections, and other subacute or chronic infections failed to demonstrate an initial fall of properdin from normal. All recovered.

Sixty-two patients with malignant blood dyscrasias have been studied (Hodgkin's disease, sarcoma, lymphocytic, myelogenous and monocytic leukemia, and multiple myeloma). Patients with active disease in each of the categories generally had levels below two units. In contrast, normal levels of properdin were observed in patients whose Hodgkin's disease, myelogenous leukemia, and sarcoma were quiescent. Reactivation of disease was associated with fall to zero levels. Patients with lymphocytic leukemia had levels below two units regardless of activity of disease.

Total complement determinations were made in 32 of the diseased patients. Serial changes in concentrations of properdin and total complement were unrelated.

Radioactive Sodium for the Measurement of Myocardial Blood Flow. BERNARD A. BERCU, WILLIAM H. DANFORTH, ERNEST E. PUND, JR., and GERALD A. DIETTERT, St. Louis, Mo. (Introduced by John R. Smith).

Na^{22} was injected into the myocardium of the open chest dog to determine if the disappearance curve could be used as a measure of myocardial blood flow. This project was predicated on the fact demonstrated by Kety that a small deposit of isotonic sodium chloride injected into striated muscle is removed at a rate that is proportional to the local blood flow. An amount of 2.5 to 3.75 microcuries in a volume of 0.1 to 0.15 ml. was injected. The radioactivity of the Na^{22} was measured at 30 second intervals with a collimated scintillation counter placed over the radioactive sodium depot. Counts per minute were plotted against time on semilogarithmic paper. Coronary blood flow was measured with a flowmeter connected between the left carotid artery and the cannulated left coronary artery. The myocardium supplied by the left coronary artery was determined by the postmortem injection of Evans blue dye into the cannula in the left coronary artery. The muscle colored by the dye was then weighed.

The slope of the decrease in the local radioactivity of the injected Na^{22}, k, was determined by the formula:

$$k = \frac{C_0 - C_1}{0.434\ (t_0 - t_1)}$$

where C_0 and C_1 are the logarithms of the radioactive counts at the times t_0 and t_1, respectively.

It was found that the slope of the disappearance curve, k, was a measure of myocardial blood flow. This value changed rapidly if blood flow was altered. With no coronary blood flow, as in the fibrillating heart, the k value is zero. Studies of the effect of work load and heart rate on the k value are underway, but these do not seem to alter it.

The Anabolic Effect of Sheep Prolactin in Man. DELBERT M. BERGENSTAL* and MORTIMER B. LIPSETT, Bethesda, Md.

Growth hormone prepared from the pituitaries of animals other than primates has been shown to be inactive in man. In contrast to this finding, we wish to report the anabolic effects of sheep prolactin in man. The preparation used was provided by the Endocrinology Study Section of the Public Health Service and had a potency of 20 International Units per milligram as determined by the pigeon crop-sac assay. There was only slight contamination with adrenocorticotrophic hormone, thyroid stimulating hormone, follicle stimulating hormone, and luteinizing hormone.

This preparation was given in 25 mg. doses twice daily subcutaneously to four women who had been subjected to hypophysectomy. The urinary nitrogen excretion decreased from 1 to 3 grams daily without significant change in fecal nitrogen. This effect took two to three days to develop and remained two to three days after discontinuing the prolactin injections. The urinary amino acid nitrogen was elevated during the periods of prolactin administration. Potassium and phosphorus balances were generally positive during periods of positive nitrogen balance.

Prolactin was ineffective in causing nitrogen retention in one man and in one woman who had had a pituitary stalk section and was lactating. Lactation did not increase in this patient nor was it noted in any of the other patients. There were no changes in the urinary excretion of 17-ketosteroids and 17-hydroxy corticoids throughout the studies.

In order to rule out the possible effect of the small amount of sheep growth hormone contaminating the prolactin, a purified sheep growth hormone was given to one of the patients who responded to prolactin. At a dose 10 times the maximum contaminating amount, the nitrogen balance and urinary amino acid nitrogen were unchanged.

These experiments demonstrate that a purified fraction of sheep pituitary which is active in stimulating the pigeon crop-sac can cause nitrogen retention in the hypophysectomized woman.

Granulocytic Activity of Human Plasma. HOWARD R. BIERMAN,* G. JUNE MARSHALL, TADASHI MAEKAWA, KEITH H. KELLY, and PAULINE PLANTE, Duarte, Calif.

The uniform response of leukopoiesis in all parts of the widely distributed red marrow indicates control by circulating substances. If humoral control of granulocytopoiesis exists, the plasma obtained during marrow regeneration following a nondestructive period of leukocyte depletion should exhibit granulocytic activity.

The intraperitoneal injection of 1 ml. of plasma per 100 gram of rat (Sprague-Dawley or Wistar) causes a characteristic absolute granulocytosis during the first 10 hours. Employing this rat bioassay system to measure the granulocytic potency of plasma, 30 subjects were studied on 71 occasions.

Fourteen plasma samples from 10 apparently healthy laboratory personnel induced an absolute granulocytosis of 215 to 663 per cent of the control level. Heating to 56° C. for 195 minutes or freezing at 4° C. for 6 weeks resulted in loss of activity. Nineteen plasma samples of 7 patients with nonhematologic neoplastic diseases induced granulocytosis of 88 to 893 per cent of the control level.

Leukopheresis in dog and man is followed by an increased granulocytopoiesis within 72 hours. Following 10 leukophereses in 6 patients, the plasma obtained 48 to 96 hours thereafter induced an absolute granulocytosis of 392 to 1,460 per cent of the control level and was distinctly higher than the granulocytic activity of plasma obtained before or 24 hours after leukopheresis.

Sixteen plasma samples from 10 patients with granulocytic and lymphocytic leukemia exhibited granulocytic activity (278 to 1,073 per cent). Four of the five patients whose plasma showed the highest granulocytic activity had acute myeloblastic leukemia. The granulocytic response to leukemic plasma was usually more prolonged than observed with nonleukemic plasma.

Further studies characterizing the granulocytic activity of human plasma indicate that it is related to the state of granulocytopoiesis in the marrow, is variable in its potency from day to day, and is thermolabile.

"Low Pressure" Kidney and the Water Concentrating Mechanism. WILLIAM D. BLAKE,* Portland, Ore.

A number of investigators have observed that moderate reduction in glomerular filtration rate (GFR) may be associated with either increased or decreased osmotic concentration of the urine. The direction of change apparently depends on whether flow is partially decreased in all nephrons or completely blocked in only a fraction of the nephron population. The above observations have been confirmed and extended, particularly with respect to complete cessation of flow in a portion of the nephrons. Studies were carried out on anesthetized, laparotomized dogs in which urine was collected separately from the two kidneys by ureteral catheterization. Creatinine clearance was used to estimate GFR. Urinary osmotic pressure (freezing point depression) was established above or below that of plasma by the intravenous infusion of hyper- or hypotonic solutions. After two or three "control" collection periods GFR was acutely decreased in one kidney either by partial occlusion of the main renal artery or by complete occlusion of a branch thereof. Partial occlusion of the main artery increased osmotic pressure of urine whether control urine samples were hyper- or hypotonic to plasma. When complete cessation of flow to a portion of the nephrons was induced by clamping a branch of the renal artery, there was an increase in GFR/TmG, urine flow/GFR and osmolar clearance/GFR ratios. The osmotic pressure of urine decreased when control samples were hypertonic to plasma and showed inconsistent and probably insignificant changes when control samples were hypotonic. Presumably complete cessation of blood flow to part of the kidney led to increased GFR in remaining functional units. An increased solute load per tubule would diminish osmotic concentration of a hypertonic urine. Physiological significance of this means for diminishing GFR remains to be explored.

Hypothalamic Irradiation in the Rat. P. BLANQUET and C. A. TOBIAS, Berkeley, Calif. (Introduced by John H. Lawrence).

The hypothalamus has been found to exert a control over thyroid metabolism. This important fact has usually been shown by the use of stereotaxic lesions produced in the median eminence. The possibility of irradiating strictly localized sections of the rat hypothalamus by means of a focused beam of high energy particles (from the Berkeley 184 inch cyclotron) has recently been demonstrated, and we have been able to focus the beam on different areas of the median eminence.

All rats (15 animals) whose median eminences were irradiated manifested localized cleancut damage at this level. The uptake of I^{131}, determined *in vivo* and after dissection of the thyroid, was normal or slightly elevated compared with controls. However, the amino acid content of the gland was found to be greatly altered. The intrathyroidal concentrations of monoiodotyrosine and diiodotyrosine, precursors of the hormone, were normal, but thyroxine was very low in all our experiments (between one-third and one-tenth of its mean value in blanks). This finding indicates that it is possible to obtain selective effects on thyroid metabolism even in the absence of pituitary lesions and seems to suggest a precise control by the median eminence area over the synthesis of thyroxine.

Acid Mucopolysaccharide Content of Thyroid Gland in Thyroid Disease and After Thyrotropin. ALFRED J. BOLLET and WILLIAM H. BEIERWALTES, Detroit and Ann Arbor, Mich. (Introduced by Stefan S. Fajans).

Thyroid tissue has been reported to be rich in glycoprotein and poor in mucopolysaccharide. Acid mucopolysaccharide analyses were done by a new chemical method on human and dog thyroid and the skin of patients with pretibial myxedema. Thyroids of untreated dogs showed an average mucopolysaccharide content of 64.6 μg. per cent dry weight measured as uronic acid (carbazole method). After the administration of 20 to 30 units of thyrotropin (TSH) to 3 dogs, the concentration increased 1.6- to 2.3-fold compared to control lobes. It is of interest that the concentration of mucopolysaccharide in the thyroid increased under TSH stimulation, when cell mass increased and intrafollicular colloid decreased.

These observations on the dog thyroid after increased TSH activity prompted us to perform mucopolysaccha-

ride assays on skin and thyroid tissues in human thyroid diseases allegedly associated with varying degrees of TSH activity. Biopsy tissue from areas of pretibial myxedema in three patients contained 2.4 to 3.7 times as much mucopolysaccharide as clinically uninvolved areas of skin from the same patients. The mucopolysaccharide content of surgically removed thyroid tissue from five patients with colloid adenomatous goiters ranged from 125 to 288 mg. per cent; two patients with exophthalmic goiter and one with Hurthle cell adenoma had concentrations in the same range. The highest value obtained, 421 mg. per cent, was in a patient with Hashimoto's struma. Approximately 60 per cent of the mucopolysaccharide in these glands was digested by testicular hyaluronidase. Chromatographically at least two components were present. One had electrophoretic and chromatographic characteristics of chondroitin sulfate and the other of hyaluronic acid. Further specimens are under assay at present to add statistical significance. Appropriate histology correlation will be made.

A Measurement of the Effect of Psychic Stimulation on Peripheral Venous Tone. STUART BONDURANT, SANFORD I. COHEN, and ALBERT J. SILVERMAN, Wright-Patterson AFB, Ohio, and Durham, N. C. (Introduced by William M. Nicholson).

Evidence of psychogenic changes in peripheral vascular tone is often observed. The present study was designed to measure these changes in the tone of peripheral veins.

Pressure was recorded from a temporarily occluded, tributary-free forearm vein segment. Pressure change in this system is caused by neurogenic change in the tone of the vein. This pressure was measured as each of 10 subjects heard a randomized series of four "charged" words, four "bland" words and four equivalent periods of silence. The subjects were alone in a dark, soundproof room throughout the experiment. The words were delivered at four minute intervals through earphones. Venous occlusions were released for two minutes between measurements. The "charged" words (words which were expected to shock the subject) and the "bland" words (words to which the subject was expected to be indifferent) were matched in number of syllables and were delivered at equal volumes.

The group mean maximal pressure changes were: following a period of silence, +2.7 cm. H_2O (range, −3 to +17); following a "bland" word, +6.9 (−1 to +24); following a "charged" word, +11.6 (+1 to +32). The large ranges of segment pressure change chiefly reflect differences among individuals in the magnitude of the response. The difference between the response of the group to each of the three conditions is significant at the $p < 0.002$ level (nonparametric analysis). The pressure changes occurred slowly, reaching a peak 10 to 15 seconds after the stimulus was given, and persisted, under the experimental conditions, for 30 to 60 seconds. The pressure change following a "charged" word was of the same order of magnitude as that observed in the forearm vein segment following Valsalva maneuver.

Such psychogenic change in peripheral venous tone would be expected to influence circulatory dynamics in health and disease and would probably modify various clinical and experimental tests of cardiovascular function.

Ventricular Rate and Response to L-epinephrine and Levarterenol in Experimental Heart Block: The Influence of Pentobarbital, Hydrocortisone, and Acid-Base Changes. SAMUEL H. BOYER and ARTHUR W. CHISHOLM, Baltimore, Md. (Introduced by Victor A. McKusick).

Mortality in Stokes-Adams disease remains high, death occurring when an adequate myocardium might respond to proper stimulation. Some factors which may influence instability of the ventricular pacemaker and response to drug therapy have been investigated. Levarterenol and *l*-epinephrine (0.0045 micromoles per kilogram) were rapidly injected intracavally in dogs with surgically induced chronic complete heart block. In trained animals the change produced in ventricular rate was inversely related to resting rate. The absolute rate, however, did not depend on resting rate and was fairly fixed for a given drug dosage in each dog. Therefore the modification of resting rate and response to sympathomimetic amines could be studied independently and simultaneously.

Intravenous pentobarbital was followed by transient increase in resting rate and significant ($p < 0.02$) persistent increased drug sensitivity. Intravenous hydrocortisone had no acute effect on either parameter. Acidosis ($pH \leqq 7.1$) produced marked slowing of resting rate and refractoriness to sympathomimetic amines. CO_2 was a much more powerful depressant of resting rate than was 0.6 N HCl for the same arterial pH. Drug responsiveness was depressed equally by both respiratory and metabolic acidosis. Alkalosis ($pH \geqq 7.5$), however induced, was accompanied by roughly parallel increases in resting rate and drug sensitivity. Ectopic beats following sympathomimetic amine injection were observed principally during periods of decreasing alkalosis.

These observations suggest a few ways in which ventricular rate and response to sympathomimetic amines may be altered. Response to *l*-epinephrine and levarterenol is apparently dependent upon extracellular pH while resting rate, depressed more by CO_2 than by mineral acid, is possibly dependent upon intracellular pH. Increased ventricular rate with elevated pH may be due to hypersensitivity toward endogenous sympathetic discharge. Acidosis, particularly of respiratory origin, should be promptly recognized and treated in the patient with complete heart block. When no initial acid-base derangement is present, alkalinization is hazardous.

Saluretic Effect of Antidiuretic Hormone (ADH) Inhibition in the Hydropenic State. JOHN W. BOYLAN and DOROTHY E. ANTKOWIAK, Buffalo, N. Y. (Introduced by David K. Miller).

When conditions known to enhance water diuresis are imposed during hydropenia, we have found that a different pattern of solute excretion is observed.

The methods employed were *1*) negative pressure breathing (NPB), and *2*) alcohol, both of which have been shown previously to increase urine flow by an increment of free water, solute excretion being relatively unaffected. In NPB the postulated mechanism is one of reflex inhibition of ADH secretion initiated by stretch volume receptors within the thorax. Alcohol is believed to inhibit ADH secretion by direct action on the neurohypophysis.

The subjects were male medical students, known to respond by diuresis to NPB. They remained supine, urine being collected by an indwelling catheter. Calculations of osmolar clearance (C_{osm}) and free water excretion (C_{H_2O}) were made in the usual way from the determined osmolar concentrations of urine and plasma.

When three consecutive half-hour periods revealed a steady state of urine formation, the subjects breathed at a continuous pressure of -15 cm. H_2O for one-half hour. A period of recovery followed.

Under these conditions the initial increase in urine flow is associated with an increase in solute excretion in such a way that the free water excretion actually falls below control levels.

This finding was duplicated in each subject when alcohol ingestion was substituted for NPB. The alcohol was given as 100 proof bourbon whiskey, 45 to 60 ml. by mouth, the amount found to produce a diuresis of comparable magnitude to that following NPB.

These observations suggest a diphasic consequence of ADH inhibition at low urine flows, the initial action being one of saluresis. Alternate mechanisms will be discussed, but the data indicate that volume receptor activity in the usual physiological state of water conservation is more effective than studies carried out during hydration have indicated.

The Uptake of Isotopic Magnesium in Different Portions of the Heart. J. Leonard Brandt, Warren Glaser, and Audrey Jones, Brooklyn, N. Y. (Introduced by R. Janet Watson).

Previous studies from this laboratory have shown that the uptake of Mg^{28} (half-life, 21.3 hours) per gram dry tissue is from 3 to 10 times greater in heart muscle than in skeletal muscle over a 48 hour period of observation. The studies focused attention on the unusual "avidity" of heart muscle for isotopic magnesium as compared to skeletal muscle since the reported magnesium content of each is about the same. The present study is an extension of these observations.

In a series of 4 dogs and 6 rabbits which received isotopic magnesium intravenously and were sacrificed at intervals over a 48 hour period, samples of muscle from the cardiac septum, left ventricle and right ventricle were taken and the concentration of Mg^{28} per gram dry weight determined. The concentration was highest in the septum and least in the right ventricle in 8 of the 10 animals. According to the binomial distribution, the probability of any sequence of 3 out of a possible 6 occurring 8 out of 10 times by chance alone is about 1 in 8,000. Combining the data for all 10 animals, the t test for the significance of the differences between the means of related samples is significant at $p < 0.001$ for comparison of septum with left ventricle, septum with right ventricle and left ventricle with right ventricle. Thus, the sequence septum, left ventricle, right ventricle, as regards the order of concentration of Mg^{28} in the heart, has very high statistical significance.

The mean values of the per gram dry weight concentration of Mg^{28} remain different in the samples over the 48 hours, and are 0.0295, 0.0269, and 0.0207 μc. for septum, left ventricle, and right ventricle, respectively. Reported differences in the per cent muscle and connective tissue in the right and left ventricles are in the same direction (more muscle per gram on the left) as the concentration differences of Mg^{28} in the right and left ventricles. However, the anatomical differences between right and left ventricles are of a greater order of magnitude than the observed chemical differences, and therefore do not completely explain the findings. It is concluded that the finding of the highest Mg^{28} concentration in the cardiac septum, least in the right ventricle with the left ventricle intermediate, has biological significance.

The Mechanism of Tolerance to Fever. Abraham I. Braude,* Margaret Zalesky, and Herndon Douglas, Pittsburgh, Pa.

The state of diminished febrile reactivity, or tolerance, which follows repeated injections of bacterial pyrogens has been associated with accelerated clearance of circulating pyrogen as measured by the transferable-pyrogen technique of Beeson, or by our method employing massive doses of radioactive endotoxin. As high-activity Cr^{51} became available, however, we repeated our clearance studies with smaller doses approaching those used in fever studies, and injected 0.1 mg. Cr^{51}-labeled *E. coli* endotoxin intravenously. Tolerant and nontolerant rabbits cleared this dose of endotoxin at equal rates with circulating concentrations falling 90 per cent within 15 minutes. To confirm these results with another pyrogen, tuberculous rabbits were made tolerant to tuberculin-fever by repeated intravenous injections of 1.0 mg. tuberculin (PPD). After intravenous injection of 5.0 mg. Cr^{51}-labeled PPD, circulating tuberculin disappeared at identical rates in nontolerant and tolerant tuberculous rabbits, with 80 per cent cleared at 30 minutes. This dissociation of enhanced clearance from febrile tolerance also occurred in rabbits whose tolerance to the pyrogenic action of endotoxin disappeared; such animals cleared massive doses of endotoxin much faster than controls despite equal febrile responses.

Turning elsewhere for explanations of tolerance, we studied febrile responses after two successive injections of endotoxin at various intervals. At two hours, a second injection was met by complete refractoriness; but this refractory state gradually diminished until a monophasic febrile response appeared at 12 hours that was identical to the classical tolerant fever curves ordinarily requiring

a week to induce by daily injection. Rabbits injected repeatedly with 0.001 mg. endotoxin every 12 hours maintained the refractory monophasic response and the tolerant pattern. This refractoriness at 12 hours was unaccompanied by accelerated removal of massive doses of circulating endotoxin.

These results indicate that febrile tolerance is independent of accelerated removal of pyrogens by reticuloendothelial cells and may represent, instead, perpetuation of the refractory state of the heat centers.

The Nature of the "Defect" in Concentrating and Diluting Mechanisms of the Diseased Kidney. NEAL S. BRICKER, RICHARD R. DEWEY, HERBERT LUBOWITZ, and JAMES M. STOKES, St. Louis, Mo. (Introduced by Herman N. Eisen).

Patients with chronic renal disease are characteristically unable to elaborate concentrated urine. Ability to dilute the urine persists longer, but ultimately diminishes and permanent isosthenuria supervenes. Although this sequence has never been adequately explained, it has been widely held that specific tubular sites for concentration and dilution are damaged by the pathologic processes. The present studies do not support this thesis.

The concentrating and diluting mechanisms were investigated in dogs with experimentally induced chronic renal disease. The lesions were either: *a*) contracted kidney histologically resembling advanced Bright's disease (produced by perfusing the anoxic kidney with an aminonucleoside solution); or *b*) progressive pyelonephritis. Employing a bladder-splitting technique, the function of the diseased kidney was compared with that of the contralateral intact kidney in serial studies.

Despite marked decrease in renal mass, both concentrating ability (solute-free water reabsorbed per 100 ml. of glomerular filtrate [$T^c_{H_2O}$/GFR]) and diluting ability (free-water clearance per 100 ml. of glomerular filtrate; [C_{H_2O}/GFR]) were consistently normal for the diseased kidney. Simultaneous values for diseased versus intact kidneys revealed $T^c_{H_2O}$/GFR to be slightly greater for the intact kidney, and unexpectedly C_{H_2O}/GFR was slightly greater for the diseased kidney. Hence, from maximum concentration through maximum dilution, urine from the diseased kidney contained relatively more free-water per unit volume of glomerular filtrate. Accordingly, maximum osmolar U/P ratios were less for the diseased kidney.

From the experimental data, it is postulated that patients with chronic bilateral renal disease develop isosthenuria because of an adaptive change in nephron function (increased GFR per functioning nephron) evoked by the decreased total nephron population, rather than from specific impairment of tubular transport systems. Under these conditions, *normal concentrating and diluting mechanisms* brought to bear on increasing volumes of filtrate would produce progressively smaller modifications of final urine osmolarity. The emerging concept furnishes a rational explanation for the observation that concentrating ability disappears before diluting ability.

Evidence for the Presence of More Than One Mechanism of Acid Production in the Kidney. WILLIAM A. BRODSKY * and JOHN T. KAIM, Louisville, Ky.

If secretion of hydrogen ions were the sole mechanism for acidification of urine, one would predict that pCO_2 of urine would be greater than or equal to that of plasma during formation of acidic urine. However, such a prediction was not verified experimentally in dogs under conditions of both metabolic and respiratory acidosis. In short, urine pCO_2 was less than plasma pCO_2 by 6 to 40 mm. of Hg in 25 per cent of over 200 periods of production of acidic urine, and was greater than plasma pCO_2 in 60 per cent of such periods. Moreover, injection of large amounts of a potent preparation of carbonic anhydrase failed to obliterate gradients in acidic urine. Similar injections did obliterate gradients in alkaline urine. The validity of calculated values of pCO_2 in several specimens of acidic urine and plasma was confirmed by tonometric determinations of $\alpha\ CO_2$ and pK'_a of these fluids. Implications of these data are: *1*) There are at least two tubular mechanisms for acidifying urine —one which tends to increase the urinary pCO_2, like H^+ ion secretion, and another which decreases urine pCO_2, like reabsorption of bicarbonate ion as such. *2*) The existence of pCO_2 gradients suggests that the permeability coefficient of certain distal tubule walls for CO_2 must be less than the value usually assumed on the basis of Krogh's datum on a rubber membrane. *3*) "Delayed" reaction rates of $CO_2 \leftrightharpoons H_2CO_3$ in lumen fluid can account for pCO_2 gradients in alkaline, but not in acidic urine. *4*) Mechanisms responsible for production of acidic urine (H^+ ion secretion or HCO_3^- ion reabsorption) are not active during production of alkaline urine.

Comparative Hemodynamic Effects of Isoproterenol and Exercise (Walking) in Cardiac Patients. ROBERT A. BRUCE,* LEONARD A. COBB, SHIGEAKI KATSURA, and JOHN MORLEDGE, Seattle, Wash.

Preliminary studies on hemodynamic effects of isoproterenol and exercises have been made on eight nonsedated but symptomatic ambulatory patients with either congenital or rheumatic heart disease. Both radial and pulmonary arteries were catheterized for pressure recording and determination of cardiac output by direct Fick principle; indicator dilution curves were obtained in many patients. Observations were made during steady states of rest, both recumbent and sitting, during intravenous infusion of isoproterenol, usually 1 to 3 gamma per minute, and during exercise while walking on a treadmill at 10 per cent grade and 1.7 mph. This exercise increased oxygen consumption about fourfold.

In each case, heart rate and cardiac index increased, as systemic resistance fell, with either exercise or isoproterenol. In confirmation of positive inotropic effect reported by Dodge and Murdough, stroke index increased with isoproterenol in all but two patients. This increase exceeded that observed with exercise. Stroke index fell with either isoproterenol or exercise in two individuals

(complete A-V block in one, mitral stenosis in the other), presumably due to shortened diastolic filling period associated with marked acceleration of heart rate. As appearance and mean transit times shortened, central volume increased in certain patients with isoproterenol. Total pulmonary resistance decreased significantly in all with isoproterenol; from the available data this reduction was chiefly postcapillary.

During recumbency the positive inotropic effect of isoproterenol, when not precluded by tachycardia, exceeds the increase in stroke index observed with moderately severe exercise in the upright posture in cardiac patients. Mechanisms of inotropic action are not known, but changes in vascular resistance and venous reservoirs may be contributory.

Coarctation of the Abdominal Aorta with Stenosis of the Renal Arteries and Hypertension: Clinical and Pathological Study of Two Cases. ALBERT A. BRUST,* JOHN M. HOWARD, MILTON BRYANT, and JOHN GODWIN, Cincinnati, Ohio, and Atlanta, Ga.

Two youthful hypertensive patients with stenosis of the abdominal aorta and renal arteries, both of whom came to necropsy, have afforded an opportunity for detailed study of the associated clinical and pathologic findings. Because of the experiences with the first patient, a correct diagnosis was made antemortem in the second.

Although these patients bore striking similarity to each other their clinical manifestations were clearly distinguishable from those of thoracic coarctation. In both, symptomatic hypertension appeared at a youthful age, systolic and diastolic blood pressure elevation in the legs equaled or exceeded that in the arms, a systolic thrill and bruit were present over the upper abdomen and lumbar spine, and pulses in the lower extremeties were only slightly diminished in volume. The ribs were not notched and collateral circulatory channels were not apparent.

Urinalyses, concentrating capacity and phenolsulphonephthalein excretion tests were normal. Aortography clarified the diagnosis in both instances but a complication of the procedure caused the death of one of the patients. The other died of cerebral hemorrhage.

Pathologic features included stenosis of the abdominal aorta at the renal artery level and partial obstruction of both renal arteries from intimal thickening, apparently occurring as a "jet stream" phenomenon. There was no evidence of hypertensive vascular or parenchymal renal damage distal to the constricted arteries. In addition, the aorta of one patient contained an anomalous valve-like structure 2 cm. in length which produced partial aortic obstruction above the renal arteries.

Twelve reports of coarctation limited to the abdominal aorta have appeared in the medical literature since 1861. Their manifestations plus those of these two additional patients facilitate the delineation of certain characteristic physical signs whose clinical recognition is essential to accurate diagnosis and the planning of appropriate surgical therapy.

Factors Influencing the Diuretic Response to Ingested Water. MAURICE B. BURG, SOLOMON PAPPER, and JACK D. ROSENBAUM,* Boston, Mass.

Water diuresis was established in normal human subjects by the institution and maintenance of a large oral water load (20 ml. per kilogram body weight). Total urine flow and free-water clearance were found to vary with osmolal clearance and sodium excretion. Thus in the seated subject, each fell proportionately, urinary osmolality remaining constant. The assumption of recumbency caused an increase in each, again proportionately, without change in urinary osmolality. Similarly a proportionate rise in free-water clearance and osmolal clearance was produced in the seated subject by the hypotonic expansion of the extracellular fluid volume. The rate of excretion of urea and potassium was unrelated to these changes. The fact that sodium excretion and osmolal clearance always paralleled each other made it impossible to correlate urine flow and free-water clearance more closely with one than with the other. Accordingly experiments were performed in which osmolal clearance was increased under conditions such that sodium excretion was not increased (seated posture, previous dietary restriction). This was accomplished either by the ingestion of urea or the infusion of mannitol, sufficient water being administered to prevent a rise in serum osmolality and antidiuretic hormone release. Under these circumstances free-water clearance did not rise despite an increase in osmolal clearance. On the other hand a comparable or lesser increase in osmolal clearance, accompanied by a rise in sodium excretion, was always associated with a rise in free-water clearance, suggesting that the renal handling of sodium is the prime mover.

Chemical Composition of Amyloid. EVAN CALKINS and ALAN S. COHEN, Boston, Mass. (Introduced by Walter Bauer).

Present studies have attempted to obtain a clear concept of the chemical nature of amyloid, and to isolate this substance in a form suitable for more precise definition.

Livers and spleens, obtained immediately post mortem from eight patients with various types of amyloidosis, and from individuals without amyloidosis, were frozen and subsequently homogenized and analyzed for dry weight, nitrogen, sulfur, phosphorus, ash, hexosamine, hexose, uronic acid, lipid, proline, hydroxyline and glycine content. Concentrated preparations of amyloid were obtained by differential centrifugation with sucrose and polyvinylpyrollidone, and were similarly characterized. Extraction of amyloid with 0.02 N NAOH yielded a fraction suitable for ultracentrifugal and electrophoretic characterization.

The results, taken together, indicate that amyloid consists of carbohydrate (5 per cent), ether soluble material (15 per cent), and protein, and has a high water content. The carbohydrate consists of hexosamine (2 per cent chiefly glucosamine) and hexoses (galactose, glucose, mannose, fucose). Sialic acid is also present. Uronic acid is less than 0.1 per cent (by orcinol and carbazole

techniques), indicating that amyloid does not contain quantitatively significant amounts of polyuronides, such as hyaluronic acid or chondroitin sulfate. The low hydroxyproline content (less than 1 per cent) suggests that the protein component is not collagen. This was substantiated by proline and glycine analyses.

Physical characterization of the extracts showed considerable homogeniety in the ultracentrifuge (sedimentation constant 6 S) and by electrophoresis (an α globulin). These values are also characteristic of serum α-2 glycoprotein, which was markedly elevated in three patients with secondary amyloidosis. The serum of one of these patients was fractionated (by Dr. Karl Schmid), and the purified α-2 glycoprotein was found to contain 4 per cent hexosamine, 5 per cent sialic acid, and 4 per cent hexose (galactose, mannose).

Renal and Cellular Responses to Acute and Chronic Respiratory Acidosis. NORMAN W. CARTER, DONALD W. SELDIN,* and H. C. TENG, Dallas, Texas.

Renal and cellular compensations for respiratory acidosis were studied in tube-fed rats kept in a metabolic cage constructed to maintain a given atmospheric CO_2 tension constant. Blood and urine electrolytes and acid-base patterns were measured; renal carbonic anhydrase and glutaminase activities were assayed. Rats were sacrificed in groups at intervals from one hour to 14 days to distinguish acute and chronic effects.

After one hour in 10 per cent CO_2 there was a rise in plasma pCO_2 (35.4 ± 2.6 mm. Hg to 91.4 ± 3.1 mm. Hg) and CO_2 content (24.7 ± 1.8 mEq. per L. to 34.4 ± 1.8 mEq. per L.) while there was a fall in plasma chloride (108.8 ± 2.8 mEq. per L. to 96.0 ± 2.5 mEq. per L.) and blood pH (7.43 ± 0.02 to 7.17 ± 0.04). By 24 hours, at the same plasma pCO_2, CO_2 content rose to 42.0 ± 3.0 mEq. per L. while plasma chloride fell to 88.1 ± 4.0 mEq. per L. No further significant changes supervened thereafter.

Urine ammonia, titratable acid and chloride excretion were increased during the first day only, while potassium excretion remained elevated throughout. Sodium excretion was unchanged. Despite profound acidosis, activities of renal glutaminase and carbonic anhydrase did not increase. Muscle analysis disclosed slight, but significant, decrease in potassium and a more marked decrease in sodium. Acetazolamide (Diamox®) administration, sufficient to suppress measurable renal carbonic anhydrase activity completely, resulted in high plasma pCO_2 (180.7 ± 31.8 mm. Hg) and a CO_2 content of 51.3 ± 4.40 mEq. per L.

From these studies it is concluded that: *1*) Elevation of bicarbonate and reduction of chloride in plasma is maximal at 24 hours; these changes are principally a consequence of cellular buffering. *2*) Renal glutaminase and carbonic anhydrase are not activated by intracellular acidosis. *3*) Increased bicarbonate reabsorption can occur as a result of hypercapnea in complete absence of renal carbonic anhydrase activity.

Left Ventricular Function at Rest and During Exercise. CARLETON B. CHAPMAN,* ORLAND BAKER, and JERE H. MITCHELL, Dallas, Texas.

Simultaneous left ventricular volume and pressure curves were recorded in five anesthetized dogs at rest and during exercise, using a biplane cinefluorographic technique at 30 frames per second. Volume was calculated from tracings from each pair of frames using a modification of Simpson's parabolic rule. The error of the method, as determined from ventricular model experiments, is less than 10 per cent.

Resting end-diastolic volume was 76.1 ± 7 cc. and that during (not after) exercise was 64.8 ± 7 cc. The difference was consistent throughout the studies and is statistically significant.

Exercise produced slight increase in stroke work but total power production per minute rose from 37 ± 8 to 55 ± 0.6 thousand Gm.-cm. per Kg. Kinetic energy, calculated from ejection and velocity curves, was 2 per cent of total work (resting) and 4 per cent during exercise. The underestimate of potential (PV) work as a result of using mean instead of integrated values was negligible at rest but was 13 per cent during exercise. The corresponding figures for kinetic energy were 35 and 51 per cent, respectively.

Variation in the heart's application of force from stroke to stroke, and the dynamic effect of bigeminy and bradycardia, are easily discernible in the volume-pressure records.

The study demonstrates the value of biplane cinefluorographic methods for obtaining "instantaneous" ventricular volume curves. It shows conclusively that ventricular end-diastolic volume decreases during exercise, that failure to calculate kinetic energy at high flows may lead to significant error, and that use of mean values for calculating ventricular power production is inadmissable where high flows are involved.

Erythropoietic Response to Repeated Phlebotomy with Iron Replacement in Man. ROBERT G. CHAPMAN and MATTHEW BLOCK,* Denver, Colo.

Measurements in the adult human with hemolytic anemia indicate the ability to increase the rate of erythropoiesis to seven times normal. What increase may be produced in the adult who is without hematologic disorder? To answer this, two adult human males were subjected to phlebotomy two to three times a week for periods of 56 and 130 days. In order to avoid erythropoietic limitation secondary to iron deficiency, these men received intramuscular iron (Imferon®) in amounts equal to that removed by phlebotomy.

Initially a fall in hemoglobin and hematocrit occurred, but after two to three weeks, these indices stabilized at two-thirds of their original values. The rate of hemoglobin synthesis, assuming negligible *in vivo* breakdown of young red cells, reached amounts of 16.4 and 13.1 grams per day (0.27 grams per kilogram body weight per day and 0.21 grams per kilogram body weight per

day, respectively). This represents only a two- to threefold increase in erythropoiesis over that required for normal replacement of aging cells prior to the phlebotomies. The average reticulocyte counts during phlebotomy, correcting for a decreased red count, were 3.6 per cent and 4.2 per cent, respectively. The plasma iron turnover measured at the end of the experiment in the subject with the greater erythropoiesis was 165 mg. per day or five to six times normal, indicating that most of the turnover was unrelated to erythropoiesis. His red cells had taken up 85 per cent of the iron tracer by the fourth day. At this time the bone marrow contained iron and showed only a 10 to 20 per cent increase in erythroid activity in comparison with the prephlebotomy marrow.

Serum Cholesterol Esters, Cholesterol Esterase, and Intestinal Cholesterol Exchange in Patients with Malignant Obstructive Jaundice. SAMUEL CHENG, ESTEBAN PINEDA, and MALCOLM STANLEY,* Louisville, Ky., and Boston, Mass.

Total and ester serum cholesterols were determined in 85 patients with malignancy. Of 39 patients severely jaundiced (mean serum bilirubin, 21.5 mg.) the mean serum cholesterol ester percentage was 22.2 (pancreatic cancer without metastases), 25.1 (with hepatic metastases), and 21.4 (hepatic metastases from extrapancreatic sites). Of 20 mildly jaundiced patients (mean, 3.1 mg.), lesser abnormalities in respective groups were: 67.4 per cent, 64.1 per cent, and 62.1 per cent. Twenty-six nonjaundiced patients (all three groups) had normal esters (mean, 71 per cent). Decreased ester percentages were proportional to increased serum bilirubins. Absolute quantities of serum ester cholesterol were reduced in jaundice. Jaundiced patients also had increased free (absolute quantities, percentages), total cholesterols to five to six times normal. In jaundice, decreased ester does not favor diagnosis of hepatocellular disease; this decrease is also usual in malignant biliary obstruction.

In serum of a patient with localized pancreatic carcinoma (serum bilirubin, 24.6 mg.; total serum cholesterol, 900 mg.; 2 per cent esters) no synthetic cholesterol esterase activity was demonstrable.

In two patients (obstructions *complete*, one above) intestinal cholesterol exchange was: *"secretion"* (normal, 2 to 2.5 Gm. per day) 403, 392 mg. per day; *absorption* (normal, 1.5 Gm. per day; 75 per cent) 171 (37 per cent), 37 (8.5 per cent) mg. per day. Exclusion from the gut of pancreatic secretions, bile with endogenous free cholesterol and bile salts markedly reduced cholesterol absorption. Proportions of cholesterol "secretion" originating from bile intestinal mucosa are unknown. Mucosal "secretions" of 400 mg. per day may be high (hypercholesteremia, other consequences of biliary obstruction, abnormally shedding mucosa). Hence, bile probably contributes 1.6 to 2.1 Gm. per day or more normally to total secretion.

Cholesterol is absorbed largely as ester; decreases in intestinal absorption were probably mainly responsible for decreased serum esters. Free biliary cholesterol regurgitation into blood was likely an important factor in increasing serum free cholesterol. Absent cholesterol esterase probably contributed to extreme serum cholesterol ester decrease in above patient.

The Effects of Aminophylline on the Coronary and Peripheral Circulation in Patients with Emphysema. RAYMOND C. CHRISTENSEN, MURRAY GRAY, HARRY ABRAMSON, CARL KOBELT, LESEM J. BAER, and HARPER K. HELLEMS,* Detroit, Mich.

Since aminophylline increases coronary blood flow and cardiac output in the experimental animal, it is generally accepted that similar effects occur with its therapeutic use in patients with cardiac and pulmonary diseases. To determine its cardiovascular effects in 11 patients with moderately severe pulmonary emphysema, coronary and peripheral circulatory dynamics were measured using cardiac catheterization techniques before and 10 to 19 minutes after the intravenous administration of 0.5 grams of aminophylline. None of the patients had angina, and they were not in heart failure at the time of study.

The drug caused the expected pulmonary response with an increase in minute ventilation, a decrease in arterial CO_2 and no change in the resting reduced arterial oxygen saturation (88.6 ± 1.59 to 89.13 ± 1.96 per cent S.E.M., $p > 0.1$). There was a slight increase in total body oxygen consumption, a widening of the systemic oxygen A-V difference from 49.3 ± 0.7 to 62.9 ± 1.2 ml. per L., $p < 0.001$, and a decrease in cardiac output from 4.31 $\pm$ 0.28 to 3.85 ± 0.30 L. per minute, $p < 0.001$. Cardiac rate increased from 92 ± 3.0 to 113 ± 5 beats per minute, $p < 0.05$. Mean brachial artery pressure decreased from 101.4 ± 4.9 to 93.7 ± 5.6 mm. Hg, $p < 0.05$. There was a decrease in stroke volume from 47.9 ± 3.1 to 34.7 ± 2.7 ml. per beat, $p < 0.001$, and left ventricular stroke work from 67.6 ± 4.4 to 45.3 ± 4.2 Gm. M. per beat, $p < 0.001$. Peripheral resistance did not change.

Coronary blood flow decreased from 92.4 ± 4.2 to 79.5 $\pm$ 3.3 ml. per 100 Gm. left ventricle per minute, $p < 0.001$. Myocardial oxygen A-V difference widened from 11.72 $\pm$ 0.57 to 13.13 ± 0.63 volumes per cent, $p < 0.001$, chiefly due to a fall in coronary sinus O_2 content from 5.44 $\pm$ 0.40 to 4.48 ± 0.30 volumes per cent, $p < 0.05$. There was no change in myocardial oxygen consumption, myocardial R. Q., or in coronary vascular resistence. Left ventricular efficiency decreased, as indicated by a fall in efficiency index from 0.35 ± 0.02 to 0.29 ± 0.02, $p < 0.05$.

It is concluded that while aminophylline improves the ventilatory status of the patient with emphysema, it simultaneously causes potential deleterious effects on the cardiovascular system.

Effect of Histamine on Nocturnal Gastric Secretion During Sleeping and Waking States. JULES COHEN, JACK M. COLWILL, NANCY DAVIS, WILLIAM KESSLER, CHARLES R. SHEPARDSON, FRANZ REICHSMAN, and GEORGE L. ENGEL,* Rochester, N. Y.

We have reported decreased gastric secretion, including decreased responsivity to histamine, during states of

withdrawal and sleep in a depressed infant with a gastric fistula. It seemed of interest to study the secretory response to histamine during sleep in healthy subjects.

Nocturnal gastric secretory patterns were studied in 28 healthy young adults by continuous Wangensteen suction. Twelve subjects were studied once; and 16 from two to six times. Sleep or wakefulness was evaluated by frequent electroencephalograms and by continuous observation. Because of difficulty sleeping with an indwelling nasal tube, 100 to 200 mg. of pentobarbital (Nembutal®) was administered intramuscularly. Histamine diphosphate (0.1 mg. per 10 Kg. body weight) or betazole (Histalog®) (1.8 mg. per 10 Kg. body weight) was injected subcutaneously once during sleep and once when awake in each experiment.

The gastric specimens were titrated electrometrically with 0.1 N NaOH, the free and total acid end points being read at pH 3.5 and pH 8.2, respectively. We determined several physiological variables but report only on total HCl secretory rates which we consider the most reliable measurement of parietal cell activity.

For each experiment the range among subjects of the mean total acid secretory rate (excluding periods of histamine stimulation) was from 0.54 to 9.12 mEq. per hour. The greatest variation from night to night in the same subject was from 2.50 to 9.12 mEq. per hour.

The secretory response to histamine (or Histalog®) was marked in both sleeping and waking states. Mean total acid secretion in a 55 minute period of maximum stimulation was 11.70 ± 1.36 mEq. (sleeping) and 9.73 ± 0.76 mEq. (waking). The difference is not statistically significant.

Thus, parietal cell responsivity in healthy sleeping subjects remains high, suggesting that sleep in such subjects is a biological state differing from the sleep-withdrawal state in certain depressive conditions.

The Effect of Induced Anxiety and Hostility on Cardiovascular Functions. J. J. COMBS, JR., G. N. BRYANT, M. D. BOGDONOFF, and J. V. WARREN,* Durham, N. C.

Although it is recognized that affect states may influence cardiovascular function, there is little information regarding the pattern of response and its mechanism of production. It may well be that such factors play an important role in determining the manifestations or even presence of organic cardiovascular disease. To study this problem, we have observed the response of 23 normal male volunteers to induced variation in affect state produced by verbal and electrical stimuli. Heart rate, respiratory rate, arterial and central venous pressure were monitored on a photographic recorder. Cardiac output was determined by the dye technique. Following control determinations, change in affect state was attempted by suggesting an endangering personal situation or by a noxious stimulus (electric shock). Further circulatory observations were made at the point of apparent maximum emotional response. Conversational material was tape recorded, including a focused interview of the subject by an objective examiner immediately after the maximum response observations. These data were then reviewed for evaluation of affect state and only later correlated with the hemodynamic response.

In 12 subjects with evidence of induced anxiety, cardiac output increased 47 per cent. Mean arterial pressure rose 11 per cent; stroke volume, 22 per cent; heart rate, 23 per cent; and peripheral vascular resistance decreased 27 per cent. In six subjects whose poststimulatory state reflected hostility, cardiac output rose 15 per cent; arterial pressure, 9 per cent; and heart rate, 17 per cent. Insignificant changes in stroke volume and peripheral resistance occurred. Five subjects were evaluated as having no change in affect state. In these individuals, cardiac output and other hemodynamic measurements failed to change significantly.

The magnitude of cardiovascular response varied directly with the evaluated intensity of the affect state, but could not be correlated with personality patterns derived from personal history. In both affect states the pattern of response was similar, and in some respects resembled that associated with epinephrine administration.

Cation Exchanges in the Heart: Relation to the Cardiac Action Potential. HADLEY L. CONN, JR.* and JOHN C. WOOD, Philadelphia, Pa.

One hypothesis relating ion transfers to the cardiac action potential assumes that the spike phase of this potential is due to sodium influx into cells, the plateau phase to potassium outflux, and the terminal fast component a result of reversal of ion flow by a "sodium pump." We have attempted to secure information pertinent to these matters through measurement of sodium and potassium exchanges in the isolated dog heart, between cardiac cells and their environment. Tracer (K^{42} and Na^{24}) techniques were used for this purpose. With arterial blood perfusing the myocardium at constant rate, "build-up" or "washout" type tracer studies were carried on for 30 to 150 minutes. Appropriate arterial and venous blood and/or myocardial samples were analyzed for K, K^{42}, Na, Na^{24}, Cl, H_2O, pH, O_2, CO_2, and hematocrit. The potassium results were best fitted to a model representing the heart as a two compartment open, series system. The sodium results also best fit this framework with an additional parallel compartment apparently representing connective tissue. Both sodium and potassium exchange per heart beat were essentially constant over the ranges of rate studied (50 to 180 per minute), with the sodium exchange rate being twice that of potassium, 0.04 and 0.02 mEq. per Kg. per beat, respectively. When the sodium rate and normal spike potential values are considered in relation to the initial hypothesis, membrane capacitance can be calculated. The value is 10 μfd per cm.^{2}, similar to values obtained by direct measurement. Assuming unchanged capacitance through the spike and plateau phases, termination of the latter phase should occur at about -25 millivolts, another prediction close to that normally observed. The findings are further compatible with the concept of a pumping mechanism exchanging two sodiums for one potassium. Finally, the findings suggest equa-

tions quantitating the relationships between ion transfers, changes in potential, and membrane conductance (permeability).

Determination of Endogenous Water Production in Renal Failure Based on Measurement of Oxygen Consumption. E. J. COWSERT, A. A. YOUNES, and Y. MORITA, Detroit, Mich. (Introduced by G. B. Myers).

Water is produced internally by oxidation of the three major foodstuffs and release of "preformed" water by tissue protein breakdown. This endogenously formed water was measured over a 24 hour period in each of six renal failure patients. Administered food consisted of about 150 grams carbohydrate only, a similar amount being given on each of several preceding days. Carbohydrate utilized was assumed to equal carbohydrate retained. Protein catabolized was calculated from external nitrogen balance corrected for plasma NPN change. The open circuit method was used for determining total oxygen consumption, two to five minute samplings being taken every one to five hours. Oxygen consumed by carbohydrate and protein utilization, calculated from established constants, was subtracted from total oxygen utilization to obtain that used by fat. From this, the amount of fat catabolized was derived.

Carbohydrate burned ranged from 83 to 174 grams per 24 hours; protein, 24 to 208; fat, 30 to 108. Water of oxidation produced from each foodstuff and preformed water released were calculated, using accepted constants. These patients produced endogenously 236, 241, 295, 333, 358, and 930 grams of water per 24 hours. Generally, magnitude of endogenously formed water correlated positively with both body size and severity of illness.

In addition, total caloric output was simultaneously measured by an established method which depends upon a reproducible relationship between insensible perspiration and total body heat production. From this, endogenously produced water was calculated, respective values being 242, 238, 294, 374, 414, and 1,045 grams per 24 hours.

The oxygen consumption method seems suitable for metabolic study in renal failure. It is felt that it is easier to use and gives somewhat more valid results than the method based on insensible perspiration. Endogenously produced water is of sufficient magnitude to necessitate inclusion in calculating water requirements in oliguria.

Growth and Form of Leukemic White Cells in Cultures Having Varying Amounts of the Natural Radioisotope of Potassium. WAYNE A. CROCKETT and REX L. HUFF,* Seattle, Wash.

Potassium-40 occurs with a natural abundance of about 0.01 per cent and of the isotopes present in the body contributes most of the ionizing radiation. To observe its effect, leukemic white cell cultures maintained by the techniques of Osgood and Brooke have been grown in replicate preparations; the controls having potassium with a natural abundance of K^{40} while the experimental group contained only one-fifth as much K^{40} but the same amount of potassium.

The cells grown in the reduced radioisotopic concentration were more abundant and their nuclei larger. The colonies showed a lesser tendency for central pyknosis and degeneration. Cells of control preparations tended to be evenly distributed between the slide and the supernatant fluid, while the media of the experimental group contained only a small fraction of the total cells in the preparation. In the latter instance most of the cells were adherent to the slide. There was a tendency for less pleomorphism in the experimental group, suggested objectively by a lesser standard deviation of nuclear diameter.

All changes are present in the earliest harvest and examination (24 hours); however, they are significantly different at 72 hours and more pronounced thereafter. At 96 hours the diameter of nuclei of the isotopically reduced preparation is greater by 30 per cent ($p \lessapprox 0.01$) than the control group.

The effect of increased K^{40} abundance is being studied along with the use of other intracellular radioisotopes. Tissue cultures of cells derived from nonmalignant sources are being used to evaluate any unusual property of the leukemic cells.

Demonstration of the Specific Metabolic Defect in Primary Essential Hyperlipemia with Secondary Diabetes. O. B. CROFFORD, J. G. CONIGLIO, H. C. MENG, and E. V. NEWMAN,* Nashville, Tenn.

A metabolic study of 120 days duration was conducted on a patient with primary essential hyperlipemia with xanthomatosis and hyperglycemia. While ingesting a low calorie, low fat diet, the patient's fasting serum lipids gradually decreased to normal and the xanthomata were resorbed. Even then, however, the patient continued to exhibit an abnormally "lipemic" response to orally administered fat. Keeping the fat intake constant, but increasing the total caloric intake of the diet resulted in a return of the patient's hyperlipemia. The patient's elevated fasting blood sugar disappeared while ingesting the low calorie diet and did not return while ingesting the high calorie diet until after the hyperlipemia had become well established. Upon resuming the original low calorie, low fat diet, and in the absence of the resorbable xanthomata, the hyperlipemia decreased much more rapidly. I^{131}-labeled triolein was given orally to the hyperlipemic patient and to normal subjects. The level of the lipid-bound I^{131} reached in the hyperlipemic patient's serum was both higher and more prolonged than normal. By comparing results when the fasting serum lipids were normal and when they were exceedingly high, we could demonstrate that the problem was truly a decreased rate of removing the labeled fat from the serum rather than a pool size artifact. Furthermore, the hyperlipemic patient was shown to have a normal rate of removal from the serum of intravenously administered I^{131}-labeled albumin bound oleic acid. Supported by a failure to exhibit a normal plasma clearing reaction in response to intravenous heparin, these data demonstrated the metabolic defect to be an inability of the hyperlipemic patient to transform

efficiently the complex glycerides into a form which is readily accessible to the tissue cells.

Dynamics of Proliferating Cell Systems of Man Studied with Tritiated Thymidine. EUGENE P. CRONKITE,* THEODOR M. FLIEDNER, JOSEPH R. RUBINI, VICTOR P. BOND, and WALTER L. HUGHES, Upton, N. Y.

Tritium labeled thymidine (T Th) is incorporated into new DNA of proliferating cells prior to mitosis affording a nuclear label ideal for autoradiographic studies. DNA labeling was employed to study the dynamics of cell proliferation in man. A selected terminal patient received 9 mc. of T Th (0.86 mc. per mg.) intravenously. Serial autoradiographic studies of bone marrow and blood were then performed. Labeled neutrophiles appeared in the peripheral blood on the third day after injection, reached a maximum in six to eight days and were rare by the twelfth day. Labeled medium and large lymphocytes, and classical monocytes were present one day after injection, reached maximum between the third and fifth day and were rare after the sixth day. Despite the reputed high turnover rate of small lymphocytes, virtually none were found labeled. In the bone marrow intense labeling of erythroid, myeloid and primitive mesenchymal elements was noted 19 minutes after injection. There was an orderly progression of maturation with dilution of the label in the more adult forms. Erythroid labeling became minimal by the third day. Myeloid labeling persisted longer. *In vitro* studies have demonstrated the presence of labeled mononuclear cells in the peripheral blood of normal men. These apparently have the capacity to synthesize new DNA and are presumed destined to divide when seeded upon the appropriate soil. The enigma of the failure of the small lymphocyte to label *in vivo* and *in vitro* has not been explained. Further application of these techniques and the development of appropriate mathematical models should make it possible to describe fully cell turnover rate, life span and fate in man.

The Capacity for Bilirubin Production as Reflected by the Concentration of Plasma Bilirubin. WILLIAM H. CROSBY,* Washington, D. C.

When severely injured patients were given massive transfusions of whole blood, a large amount of the red cells were extravasated or phagocytosed and eventually destroyed. The hemoglobin of such cells is degraded and the plasma bilirubin is increased, reflecting the conversion of hemoglobin to bile pigment. In these patients, unless there was damage to the liver or paralytic ileus, the concentration of bilirubin did not exceed 3 to 4 mg. per 100 ml. The plasma bilirubin is normally turned over about 10 times daily. At this rate the level of 3 to 4 mg. represented a clearance of about 1.5 Gm. of bilirubin, the amount derived from the degradation of about 45 Gm. of hemoglobin. Although massive amounts of hemoglobin were available in the extravasated red cells, the rate of bilirubin formation did not appear to exceed 1.5 Gm. per day; however, the process continued for several weeks as reflected by the elevation of the plasma bilirubin. This suggested that there might be a limit to the capacity of the mechanism for converting hemoglobin to bilirubin.

The problem was studied further in healthy subjects by intravenous injections of autologous hemoglobin solutions or rapid infusions of distilled water to produce intravascular hemolysis. Plasma hemoglobin concentrations were achieved which varied initially from 50 to 500 mg. per 100 ml. The rate of clearance of hemoglobin was determined by periodic sampling and at the same time the plasma bilirubin was measured. Regardless of the dose of hemoglobin the peak concentration of bilirubin did not exceed 3.5 mg. The time of the peak was at 200 to 250 minutes after the injection.

Computations based on these results indicate that there is a limit to the capacity of the mechanism for bilirubin production, and that regardless of the quantity of hemoglobin presented the amount converted to bilirubin does not exceed 2 Gm. per hour or about 50 Gm. per day.

The Acute Effects of Carbonic Anhydrase Inhibitors on Systemic Hemodynamics. ARCHER P. CROSLEY, JR.,* CESAR CASTILLO, D. JOSEPH FREEMAN, DOUGLAS H. WHITE, JR., and GEORGE G. ROWE, Madison, Wis.

Recently, interest has been shown in the etiology of the significant reduction in glomerular filtration rate as well as the hypotension which may occur following the use of newer carbonic anhydrase inhibitors. Although chronic observations have demonstrated that such changes may occur as the result of a depletion of plasma volume, certain hemodynamic observations have occurred on such an acute basis as to preclude the latter mechanism as an etiological factor.

For these reasons combined renal and systemic hemodynamic measurements were made in eleven patients with cardiac and/or renal disease before and 60 minutes after the intravenous administration of sodium *p*-sulfamyl benzoate, dichlorphenamide or chlorothiazide. The results demonstrated that these agents are capable of producing a significant rise in urinary pH and a fall in glomerular filtration rate. Associated with the latter was a significant reduction in cardiac output which in turn appeared to be related to a decrease in venous return as evidenced by significant declines in right auricular, right ventricular end diastolic, and pulmonary artery pressures. In one patient cardiac output returned to control values following elevation of the legs.

These results are interpreted as indicating a possible causal relationship between the decline in cardiac output and glomerular filtration rate. Furthermore, the fall in the former function was related to a decrease in venous return and is therefore similar to that observed following the use of ganglionic blocking agents. Such observations may provide a clue to the acute and enhanced reductions of blood pressure which may occur following the combined use of such agents over and above that due to reduced plasma volume.

Chemical Constituents of Pine Pollen and Their Possible Relationship to Sarcoidosis. MARTIN M. CUMMINGS * and PAUL C. HUDGINS, Washington, D. C.

The geographic distribution of the birthplaces of 1,800 veterans with sarcoidosis suggested that the cause of this disease might be related to some aspect of the forest environment. In the course of laboratory studies it has been found that the pollen of pine trees in the "endemic area" has acid-fast characteristics similar to those of the tubercle bacillus. In addition it has been possible to isolate a wax containing mycolic acid and diaminopimelic acid from the pine pollen. These constituents, in combination, previously thought to be characteristic of tubercle bacilli have been demonstrated within sarcoid granuloma from clinical sources by British investigators. It is suggested (by us) that their presence within sarcoid lesions may be related to exposure to pine pollen rather than to the tubercle bacillus.

Using the methods of Anderson for fractionation of tubercle bacilli it has also been possible to demonstrate in pine pollen the presence of phospholipids, a firmly bound lipid, and "A," "B," "C," and "D" waxes similar to those found in tubercle bacilli.

Tuberculin hypersensitive guinea pigs injected intradermally with pine pollen developed epithelioidal tubercles after prolonged incubation periods in preliminary tests. Further biological studies with fractions of pine pollen are in progress.

The Measurement of Serum Sulfation Factor as an Index of Somatotropin Activity in Man. WILLIAM H. DAUGHADAY,* WILLIAM D. SALMON, JR., and FRANCE ALEXANDER, St, Louis, Mo.

It has been previously reported that sulfation factor (SF) is a somatotropin-dependent component of normal rat serum that promotes sulfate-S^{35} uptake by cartilage from hypophysectomized rats *in vitro*. SF disappears from the sera of hypophysectomized rats but is present in sera of hypophysectomized rats treated with somatotropin. *In vitro* addition of somatotropin to cartilage incubates is without comparable effect.

SF activity of normal human serum can be assayed *in vitro* by incubation of segments of costal cartilage from hypophysectomized rats in a medium containing 0.1 ml. of serum. The mean sulfate uptakes with dilutions of a normal human reference serum (obtained from a 23 year old student) are proportional to the logarithm of the amount of serum added. This relationship makes possible an estimation of SF activity of unknown sera simultaneously incubated, relative to the reference serum.

SF activity has been found at all ages. The mean and standard error for three different age groups were: eleven children (7 to 11 years), 0.84 ± 0.12; 14 adults (20 to 40 years), 0.78 ± 0.13; and 6 adults older than 50 years, 0.75 ± 0.10. The mean SF activity of sera from eight surgically hypophysectomized patients, kindly provided by Dr. O. H. Pearson, was only 0.11. Three untreated patients with active acromegaly had increased levels of SF (3.7, 4.4, and 8.2) but in five clinically inactive or extensively treated patients elevated values were observed in only two cases. SF activities below 0.35 were observed in three of six patients with nonfunctioning pituitary tumors, three patients with pituitary dwarfism and two patients with Sheehan's syndrome.

This initial experience indicates that measurement of the serum SF activity is a promising index of pituitary growth promoting activity in man.

Changes in the Impedance Locus Diagram of Swine Skin Produced by Thermal Injury. WARREN H. DENNIS and JAMES D. TELFER, Louisville, Ky. (Introduced by J. Murray Kinsman).

At the present time, there is no method for the accurate determination of the depth of thermal injury soon after injury. The electrical properties of normal and thermally injured swine skin have been studied in an attempt to develop a reasonably objective measure of the depth of burns. Measurements of potential differences and direct current resistance are subject to such wide variation that interpretation is difficult. However, changes of the impedance locus diagrams (*i.e.*, reactance versus resistance) for normal swine skin and for skin injured by applying a heated brass plate for varying periods of time were found to be consistent. The impedance of the skin as a function of frequency (5 to 20×10^8 cycles per second) was determined from lissajou patterns of the current flowing in response to a voltage of 0.5 volts p-p applied across the skin. The normal skin yielded an impedance locus diagram essentially the same as that found by others for human skin. One sq. cm. of skin can be represented by an equivalent circuit of a small resistance, 1,200 ohms, in series with a second resistance, 150,000 ohms, shunted by a polarization element. The polarization element has a phase angle of 73°. Following an incomplete thickness injury, the series resistance is unchanged while that of the parallel element is reduced to 22,000 ohms. An unexpected change occurred in the phase angle of the polarization element which became 90°. Following complete thickness injury, the capacitative reactance is completely abolished, whence the impedance locus diagram reduces to a single point on the resistance axis. By inspection of the impedance locus diagram, one can easily differentiate a complete thickness injury from an incomplete thickness injury.

Delay of Absorption of Radiolabeled Cyanocobalamin in the Intestinal Wall in the Presence of Intrinsic Factor. ALFRED DOSCHERHOLMEN and PAUL S. HAGEN, Minneapolis, Minn. (Introduced by Edmund B. Flink).

Plasma absorption curves following the oral administration of small test doses of radiolabeled vitamin B_{12} to normal subjects and patients with pernicious anemia with the addition of intrinsic factor concentrate have shown peak concentrations delayed until the eight to twelve hour interval while no or negligible activity was detected during the first three to four hours of the tests. Because the

blood samples were drawn from antecubital veins, there was a question whether the curves really reflected the rate of absorption from the intestine or whether some alteration had taken place during the passage of the blood through the liver, lungs or peripheral tissues. In order to circumvent the possible role of the liver, absorption curves were obtained in a similar manner from several patients with cirrhosis of the liver who had functional portacaval shunts. All of them exhibited absorption curves similar to those observed in the peripheral vein blood from control subjects. Because alteration of the plasma absorption curve by the passage of the blood through the lungs or peripheral tissues could not be excluded in these subjects, portal vein blood was obtained directly by catheter from another patient with cirrhosis of the liver but without portacaval shunt. A portogram immediately before and after the conclusion of the study ensured the proper location of the tube in the portal vein. The absorption pattern was similar to that described above. Therefore, the delay of plasma absorption of vitamin B_{12} in the presence of intrinsic factor apparently occurs in the intestinal wall.

Regulation of the Serum Amylase: The Effects of Alterations in Carbohydrate Metabolism in Normals, Diabetics and Some Patients with Liver Disease. DAVID A. DREILING, WILLIAM S. ROSENTHAL, and HENRY D. JANOWITZ,* New York, N. Y.

Serum amylase is generally held to be derived from the pancreas. There is reason to believe, however, that the liver may be a more important source since we have observed that glucagon, which is without effect on the exocrine secretion of the human pancreas, induces a rapid, significant, although transient depression of the levels of serum amylase. Glucagon (2 mg.) was administered intravenously to 30 normal subjects and 21 diabetic patients, and the concentrations of blood glucose and amylase determined by modifications of the Somogyi methods before and at 20 to 30 minute intervals afterwards for 2 to 4 hours. The normal subjects and the diabetic patients without evidence of exocrine pancreatic disease experienced on the average a 50 per cent fall in the level of serum amylase within 60 minutes, which paralleled the expected rise in blood glucose. Similar results were elicited by intravenous administration of 25.0 Gm. of glucose or fructose. Utilization of glucose rather than the absolute level of blood sugar appeared to be involved since depression of blood glucose produced by 15 mg. of insulin or 2.0 Gm. of tolbutamide given intravenously to six normals and five diabetic patients was accompanied by a similar significant depression of the serum amylase values, while subcutaneous administration of 2.0 mg. of epinephrine to two normal subjects induced a significant elevation of both blood glucose and amylase concentrations.

In five patients with varying degrees of hepatocellular necrosis associated with either hepatitis or portal cirrhosis, glucagon induced on the average no significant elevation of blood glucose nor any fall in serum amylase.

From these and other published studies it appears that changes in blood amylase levels may be correlated with states of altered carbohydrate utilization. The present studies suggest that the liver is the important source of the amylase normally appearing in the serum.

The Chemical Quantitation of the Daily Fecal Hemoglobin Excretion in Normals and Patients with Bleeding Alimentary Canal Lesions. FRANKLIN G. EBAUGH, JR. and WARREN BEEKEN, Hanover, N. H. (Introduced by Joseph F. Ross).

Qualitative tests for occult blood in the feces are often misleading and quantitation of fecal blood excretion by radioactive sodium chromate, though reliable, is impractical for large scale application. A simple photospectrometric test employing 1 ml. of 1 Gm. per cent of benzidine base solution, 1 ml. of 1 per cent H_2O_2, and 0.002 ml. of 1/50 aqueous homogenate of feces read at 500 mμ has been developed. Forty-six normal males excreted 1.6 ± 1.2, and 17 females 1.5 ± 1.1, ml. of whole blood per 24 hours. Ninety-nine $\pm$ 18 per cent of blood added to 73 fecal suspension *in vitro* was recovered. A recovery of 106 ± 13 per cent by the benzidine and 105 ± 10 per cent by radioactive $Na_2Cr^{51}O_4$ of 70 to 80 ml. ingested blood labeled with radioactive $Na_2Cr^{51}O_4$ was observed in 13 volunteers. Additions of $FeSO_4$ to the diet resulted in the detection of 2.3 ± 2.7 ml. more of blood in 18 24-hour fecal collections by the chemical technique than by radioactive Cr^{51} counting. $FeSO_4$ *in vitro* gave less than the equivalent of 0.1 ml. blood by the benzidine test. Heme alone was 77 per cent as reactive as when present in equivalent amounts in hemoglobin. Boiling fecal suspensions for 10 minutes decreased the amount of blood detected by 31 per cent. Of the 142 24-hour fecal collections which contained less than 3.8 ml. of blood (upper limits of normal), results of the *qualitative* benzidine dihydrochloride test were positive in 16 per cent, equivocal in 28 per cent, and negative in 56 per cent; for the 45 fecal samples which contained more than 3.8 ml. of blood, the results were positive in 42 per cent, equivocal in 36 per cent, and negative in 22 per cent. *Quantitation* of fecal hemoglobin is of potential value in the early detection of gastrointestinal bleeding lesions before X-ray changes occur. The *qualitative* benzidine dihydrochloride test is misleading 38 per cent of the time.

Steroid Metabolism in the "Salt-losing" Form of Congenital Adrenal Hyperplasia. WALTER R. EBERLEIN and ALFRED M. BONGIOVANNI,* Philadelphia, Pa.

Approximately one-third of infants with congenital adrenal hyperplasia develop hyponatremia, hyperkaliemia, and progressive dehydration. The electrolyte disturbance is often corrected only by massive doses of desoxycorticosterone and is aggravated by adrenocorticotrophic hormone (ACTH) administration. Normal or above-normal amounts of aldosterone have been found in the urine. It has been suggested that these infants secrete a salt-losing steroid or aldosterone antagonist. Failing to find such a steroid in the urine, we reinvestigated steroid excretion quantitatively.

A 24 hour collection of urine from 12 untreated patients with congenital adrenal hyperplasia and 8 control subjects was assayed for total 17-ketosteroids, for pregnanetriol by means of column chromatography, and for tetrahydrocortisone using paper chromatography. All 12 patients excreted large amounts of 17-ketosteroids and pregnanetriol, the 4 active salt-losers particularly so. Five of the 12 patients excreted "normal" quantities of tetrahydrocortisone in the urine; 3 excreted less than 25 per cent of the control; 4 excreted no detectable amount of the steroid. None of the first 5 showed any evidence of a salt-losing state; conversely, the 4 patients not excreting tetrahydrocortisone were active salt-losers. The 3 patients who excreted small amounts of this steroid had been clinically considered "potential salt-losers" because of slightly low serum sodium and moderately elevated serum potassium levels, which were corrected by cortisone treatment alone.

The present study demonstrates that in all patients with congenital adrenal hyperplasia there is inefficient synthesis of hydrocortisone. In those patients whose adrenals are able, by tremendous overactivity, to produce sufficient hydrocortisone, electrolyte disturbances do not occur. It is suggested that the salt-losing form of this disease results from an essentially complete failure of hydrocortisone synthesis due to lack of adrenal 21-hydroxylase. The present study also suggests that a minimal amount of hydrocortisone is required for aldosterone to exert its metabolic actions in man.

The Effect of In Vivo Dialysis upon Muscle Composition in Uremia. R. E. Eckel, R. S. Post, and J. H. Davis, Cleveland, Ohio. (Introduced by H. S. Ginsberg).

The effect of six hours' *in vivo* dialysis upon the water, amino acid, Na, K, Cl, and acid-soluble P content of muscle from uremic oliguric patients has been studied by means of biopsy immediately before and immediately after the procedure. Five dialyses in four patients have been studied and compared with normal findings. Muscle water was normal initially and decreased in four cases (two to abnormal values) and was unchanged in one. In three cases, extracellular water (ECF) constituted 41 to 46 per cent of total muscle water initially and intracellular water (ICW) was abnormally law. In these cases ECF decreased to 33 per cent or less of total muscle water and ICW was returned to or toward normal. In the remaining cases, ECF was less than 33 per cent of total muscle water and ECF and ICW were unchanged by the procedure. The volume of distribution of Na was smaller than that of Cl in four cases before dialysis, and remained so after dialysis in two. The most consistent predialysis finding was a high intracellular K concentration, present even though only one serum K was above 6.0 mM per L. The intracellular K concentration fell in all cases with dialysis. In two cases this was due to loss of K in excess of cell water; in the remainder, to increase in cell water without change in muscle K. Total muscle P decreased in all cases.

The Effect of Hyperventilation on EFFECTIVE Right Atrial Pressure in Man. John W. Eckstein and William K. Hamilton, Iowa City, Iowa. (Introduced by James W. Culbertson).

We demonstrated previously that active venous constriction shifts blood from the extremities during hyperventilation. This blood, if redistributed centrally, could increase atrial pressure and the availability of blood to the heart. Such a mechanism might partly explain the enhanced cardiac output observed with overbreathing.

Right atrial and intrapleural (measured with an open-ended water-filled intraesophageal catheter) pressures and the resultant (effective) pressure were registered simultaneously. Ventilation and end-expiratory CO_2 concentration were monitored in each experiment. After control values were established subjects hyperventilated for one to three minutes with maximal inspirations and passive expirations. The procedure was repeated with 5 per cent CO_2 in the inspired gas after control values returned.

Mean atrial pressure decreased in each of 12 experiments during air hyperventilation; the decrease averaged 2.9 mm. Hg. Mean intrapleural pressure fell more than atrial pressure (11 of the 12 tests) by a highly significant ($p = < 0.01$) average value of 1.4 mm. Hg. This change represents an *increase* in *effective* or distending pressure (atrial minus intrapleural pressure) within the atrium.

Mean *effective* atrial pressure also increased in seven of eight experiments during overbreathing CO_2. In each of these eight experiments the absolute level of *effective* atrial pressure was higher ($p = < 0.01$) while overbreathing CO_2 than while overbreathing air. This difference was not a function of ventilatory volume.

The *effective* atrial pressure increase while overbreathing air probably is not caused by increased atrial tone. The only other explanation is an increased volume of blood distending the chamber. The greater increase in cardiac output while overbreathing air than while overbreathing CO_2 (Burnum, Hickam, McIntosh), despite a greater *effective* atrial pressure in the latter case, may mean that diastolic distensibility of the ventricle is increased during hypocapnia.

Avoidable Errors in Selection of Patients for Mitral Valvulotomy. E. E. Eddleman, Jr., Birmingham, Ala. (Introduced by Tinsley R. Harrison).

The preoperative determination of the relative importance of murmurs in patients with mitral stenosis and other coexistent valvular defects often presents a difficult clinical problem. Some patients reveal at operation a high degree of stenosis with a minimal mitral regurgitant jet while other patients with similar clinical findings display the opposite.

It has been found that a study of precordial movements (kinetocardiograms) may be helpful in determining the relative degrees of mitral stenosis and insufficiency.

This report deals with a study of four patients in whom mitral valvulotomy might have been avoided had the pre-

cordial movement been properly evaluated. Some of the features in the precordial traces (kinetocardiograms) which have been found to be particularly valuable are as follows: *1*) A marked forward precordial movement (heave) throughout systole speaks for a right ventricular hypertrophy and hence for a predominant mitral stenosis. *2*) Exaggeration of a predominant late systolic forward movement which is ascribed to atrial filling speaks for significant mitral insufficiency. *3*) Absence of a systolic heave offers strong evidence against a functionally important mitral stenosis. *4*) A precordial heave that diminishes in magnitude in the V_3 as compared to the V_2 position associated with an isolated large apical thrust speaks for both right and left ventricular hypertrophy and hence for a significant degree of insufficiency as well as stenosis.

Induced Tolerance of Dogs to Endotoxin: Correlation of Febrile Response and Adrenal Function. RICHARD H. EGDAHL and JAMES C. MELBY, Minneapolis, Minn. (Introduced by Wesley W. Spink).

When adrenal cortical activity in dogs was determined by measuring the Porter-Silber chromogens in venous blood collected from a cannula in the adrenal vein, it was observed that endotoxin caused a marked increase in adrenal activity. Administered endotoxin causes fever and an increase in adrenal activity. Therefore, experiments were designed to determine whether dogs could be made tolerant to endotoxin so that there was no febrile response or no increase in adrenal cortical function, as determined by the minute output of 17-hydroxycorticoids in adrenal venous blood. The results were related to the dose of *E. coli* endotoxin that was used. When large doses of endotoxin (1 to 5 mg.) were administered repeatedly, only rarely did an animal fail to exhibit both fever and an increase in adrenal activity. Furthermore, these large doses given daily did not depress adrenal or pituitary function. When smaller doses of endotoxin (0.01 mg.) were given daily, tolerance quickly developed as far as the febrile response was concerned, but adrenal activity was not depressed. Tolerance to the fever-promoting effect of endotoxin lasted for only a few days after the daily injections of endotoxin were discontinued. In not a single experiment was it possible to induce an increase of adrenal activity without fever following a single dose of endotoxin. From these experiments it can be concluded that: *1*) The response of the adrenal cortex to endotoxin is not due to fever. *2*) Tolerance to the fever-promoting property of endotoxin is readily accomplished. *3*) Tolerance to the stimulating effect of endotoxin on adrenal cortical function is produced with difficulty. *4*) Repeated sublethal doses of endotoxin do not cause a measurable depression of pituitary-adrenal function.

Rates of Osteogenesis Measured by Nonradioactive Strontium in Subjects with Normal and Decreased Skeletal Mass. E. EISENBERG, T. RUSSELL FRASER, and G. S. GORDON,* with the technical assistance of JEAN MARIE SIMIEN, San Francisco, Calif., and London, England.

Others have measured rates of skeletal formation in man with bone-seeking radioisotopes and have shown that strontium moves from blood to bone at the same rate as calcium. An accurate spectrophotometric method permits use of *nonradioactive* strontium for this purpose. Absence of radiation hazards allows repeated measurements in the same person. Ten mEq. of strontium gluconate is infused, and serum and urine content are determined for four days. The size of the exchangeable calcium pool (Ca_e) and its rate of turnover are calculated from the usual dilution formulae. Fecal excretion is negligible, and tracer does not reenter the pool in this time. Subtraction of the rate of urinary loss from total daily turnover yields the rate at which calcium is incorporated into bone (Ca_b). The following values were obtained: In 12 normal subjects, Ca_e was 211 ± 11 mEq. and Ca_b 47.5 ± 2.2 mEq. per day. In 23 senile osteoporotics, Ca_e was 164 ± 5.7 mEq. and Ca_b 34.9 ± 1.7 mEq. per day. In 2 thyrotoxic osteoporotics, Ca_e was 339 mEq. and Ca_b 121 mEq. per day. In 1 osteoporotic acromegalic, Ca_e was 311 mEq. and Ca_b 126 mEq. per day. In 7 hyperparathyroid patients, Ca_e was 362 ± 28 mEq. and Ca_b 101 ± 14 mEq. per day. In repeated measurements in 7 persons, the second value for Ca_e was 99.1 ± 6.6 per cent of the initial value, and for Ca_b 93.7 ± 3.3 per cent.

Values obtained by this technique agree well with those obtained by isotopic methods, show little scatter among normal subjects, and demonstrate a reduced rate of osteogenesis in senile osteoporosis. Since osteogenesis is accelerated in thyrotoxicosis and acromegaly, the osteoporosis in these disorders probably results from increased osteolysis. Ca_b is retarded in spontaneous and induced hypercorticism. The action of estrogens and androgens in osteoporosis is under investigation.

The Influence of Blood Glucose on Renal Clearance of Phosphorus in Diabetic Patients. H. ELRICK, C. J. HLAD, JR., N. E. WHIPPLE, and Y. ARAI, Denver, Colo. (Introduced by G. Middlebrook).

Previous studies from this laboratory have shown that in normal subjects the level of blood glucose (below the renal threshold for glucose) exerts a powerful and sensitive influence on the renal clearance of inorganic phosphorus. This was demonstrated by the finding of a consistent positive correlation between blood glucose and the renal clearance of phosphorus during glucose infusion.

In the present experiments the effect of intravenous glucose on phosphorus clearance was studied in five patients with severe diabetes. Studies were carried out in the basal state 25 hours after the last dose of insulin. Renal clearance methods were used and 7 to 10 10-minute urine collection periods were obtained before and throughout the glucose infusion.

Endogenous creatinine clearance, used as a measure of

glomerular filtration rate, remained unchanged. In two patients it was normal (109 and 129 ml. per minute) and in three it was diminished (47 to 79 ml. per minute). The lowest phosphorus clearance in the control period was 31 per cent (expressed as per cent of filtered load excreted); the maximum was 50 per cent. These values exceeded the maximum (21 per cent) observed in normal subjects.

Following glucose, serum phosphorus showed a rise (mean, 16 per cent) rather than the fall commonly observed in normal subjects. Phosphorus clearance did not parallel blood glucose; indeed, in four of the five patients it decreased with increasing blood glucose levels. The remaining patient had the lowest fasting blood glucose (147 mg. per cent) and an increase in phosphorus clearance was observed.

The findings indicate that the regulatory influence of blood glucose on phosphorus clearance observed in normal subjects does not occur in the diabetic patient with markedly elevated blood sugar. This is consistent with previous observations in normal subjects with induced glycosuria. These data further support the concept that phosphorus and glucose compete in the process of renal reabsorption over a wide range of plasma levels.

Further Studies on the Effects of Estrogen and Testosterone on the Circulating Thyroid Hormone. NORMAN H. ENGBRING and WILLIAM W. ENGSTROM,* Milwaukee, Wisc.

The fact that the serum protein bound iodine (PBI) rises in normal pregnancy led to our observation that estrogen administered to persons with normal thyroid function causes an increase in PBI. Conversely, administered testosterone has been shown to decrease the PBI. The estrogen-effect is associated with an increased capacity of serum interalpha globulin to bind thyroxine. These observed phenomena prompted further investigation, since changes in PBI might result from altered thyroidal output, or altered rate of peripheral disposal of hormone.

The possible independence of estrogen-induced increase in PBI on production of hormone was assessed by administration of estrogen to athyreotic individuals on replacement therapy. In those maintained entirely eumetabolic on desiccated thyroid, a modest rise in PBI resulted; in those on pure L-thyroxine, the rise was more marked. Patients on inadequate replacement therapy exhibited no rise in PBI. This might be anticipated because the serum of hypothyroid individuals already showed an abnormally increased thyroxine-binding capacity. These observations indicate that a functioning thyroid gland is *not* essential for the estrogen-effect on PBI.

The rate of disposal of thyroxine was assessed by radioactive thyroxine survival studies in six normal individuals before and during estrogen administration. In each case, there was a prompt prolongation of survival time; the average control half-life of 6.7 days increased to 11.2 days with estrogen administration. Thus, the estrogen-effect on PBI is accompanied by initial decreased rate of utilization or turnover of thyroid hormone.

The effect of administered testosterone was studied in nine euthyroid males; in each the PBI fell. The thyroxine-binding capacity decreased in three, but remained unchanged in six. No alteration in thyroxine survival time was demonstrated in five studies. Thus, the testosterone-induced fall in PBI is not as easily interpreted as the estrogen-induced rise.

Studies on the Phylogenesis of Gamma Globulins and Plasma Cells. RALPH L. ENGLE, JR., KENNETH R. WOODS, and JAMES H. PERT, New York, N. Y. (Introduced by Paul Reznikoff).

There is evidence that the production of gamma globulins and antibodies is related to plasma cells. We have initiated studies designed to test whether or not the phylogenesis of gamma globulins parallels that of the plasma cell.

Serum proteins from several marine invertebrates, elasmobranchs, and teleosts were analyzed by zone electrophoresis in starch gel (Smithies). Proteins migrating toward the cathode at pH 9.0 were considered to be gamma globulins. Microscopic examinations were conducted to determine whether or not plasma cells could be found in the blood or organs of the marine forms. Blood smears of invertebrates were fixed in osmic acid or formalin vapor. Blood of vertebrates was smeared on slides and dried. Spleens and other organs were sectioned, and the exposed surfaces lightly touched to glass slides. These touch preparations were dried and then treated with Wright-Giemsa stain. Some fresh preparations were examined with the phase contrast microscope.

The invertebrates studied included 14 species of crustacea, the horseshoe crab, scorpion, squid, and oyster. Within this group, no gamma globulin-like protein was identified and no cells suggestive of plasma cells were found in the blood and tissues. Among the eight species of elasmobranchs studied, all had gamma globulin-like bands in the electrophoretic pattern. Cells having morphologic characteristics of plasma cells were readily found in touch preparations of the spleens. Twelve species of bony fish were examined. In this group, no gamma globulin-like protein was found. After prolonged searching, it was possible to identify a small number of cells having some of the characteristics of mammalian plasma cells in the touch preparations from the spleens. In all species studied, there was remarkable specificity in the serum electrophoretic patterns and the pattern within a given species was consistent.

In the forms thus far examined, there appears to be a phylogenetic association between gamma globulin and plasma cells.

Selective Inhibition of Renal Concentrating Ability by Experimental Hypercalcemia. FRANKLIN H. EPSTEIN,* DAVID BECK, MANUEL J. RIVERA, HOWARD LEVITIN, and FRANK A. CARONE, New Haven, Conn.

Polyuria and polydipsia are well-known clinical accompaniments of hypercalcemic states. In order to investigate their genesis, 15 white male Sprague-Dawley rats weighing 300 to 400 grams were given 200,000 to 400,000 units of vitamin D_3 in oil subcutaneously daily for four days, resulting in a rise in serum calcium of 3 to 4 mg. per cent and spotty nephrocalcinosis, distributed chiefly in the medulla, but affecting the cortex as well. Maximum urinary solute concentration tested after dehydration and vasopressin fell from an average of 2,596 mOsm. per Kg. to 1,655 mOsm. per Kg. Blood urea nitrogen, urea clearance and phenolsulfonephthalein excretion were unchanged in most instances, although when larger amounts of vitamin D_2 were given, all renal functions were depressed.

The effect of hypercalcemia of relatively short duration on the ability of the kidneys to reabsorb water free of solute was investigated in dogs given 60 to 90 units per Kg. of parathyroid hormone subcutaneously in divided doses over a 24 hour period. Serum calcium rose 4 to 5 mg. per cent. $T_m{}^cH_2O$, measured during mannitol diuresis, dropped precipitously. Two dogs excreted urine hypotonic to plasma despite a constant infusion of large amounts of exogenous vasopressin. Glomerular filtration rate fell in some dogs but marked depression of $T_m{}^cH_2O$ was seen in those animals as well in which inulin clearance remained the same or rose following parathormone. Acute infusions of calcium salts, while producing comparable elevations of serum calcium, did not depress $T_m{}^cH_2O$ to the same degree as did parathormone.

These data suggest that hypercalcemia of sufficient duration specifically inhibits the ability of the renal tubules to concentrate urine and conserve water.

Chromatographic Characterization of the Serum Protein Changes in Pathologic Sera. JOHN L. FAHEY, PATRICIA F. MCCOY, and ANN P. HORBETT, Bethesda, Md. (Introduced by Charles G. Zubrod).

Chromatographic fractionation of serum proteins into 16 or more components has been reported recently. Such fractionation, carried out on diethylaminoethyl-cellulose columns in the cold and utilizing a gradient elution system employing phosphate buffers, permits characterization of the serum proteins to an extent not previously feasible by electrophoresis or ultracentrifugation. The usual electrophoretic fractions are characteristically further subdivided into a number of separate components by this chromatographic procedure.

Modifications in the chromatographic procedure have made possible detailed examination of 1 and 3 ml. serum samples. Sera obtained from 36 patients with a variety of diseases have been examined chromatographically, electrophoretically and, in some instances, in the ultracentrifuge. In most of the disease states studied multiple serum chromatographic changes were seen and, in several, identifying serum chromatograms were obtained. Macroglobulinemic sera, although indistinguishable from multiple myeloma sera by electrophoresis, were usually easily distinguishable chromatographically. Macroglobulins are eluted later in the chromatogram than are myeloma proteins. The major gamma globulin peak was characteristically missing from the agammaglobulinemic sera. The ceruloplasmin-containing component was markedly diminished in Wilson's disease and significantly increased in several patients with Hodgkin's disease. Transferrin and albumin levels were decreased and alpha-1 and alpha-2 glycoproteins elevated in many of the sera from patients with infectious, neoplastic and collagen diseases. In selected abnormal sera the chromatographic distributions of protein-bound vitamin B_{12}, alkaline and acid phosphatases and other proteins of physiologic interest have been determined and compared with normal serum. Serum protein chromatography has been found to provide considerable information in addition to that obtainable by electrophoresis or ultracentrifugation.

Occurrence of a Sprue-Like Syndrome During Neomycin Therapy. WILLIAM W. FALOON, CURTIS J. FISHER, and KATHLEEN C. DUGGAN, Syracuse, N. Y. (Introduced by Eugene L. Lozner).

The occurrence of diarrhea during neomycin therapy in patients with cirrhosis has stimulated investigation of fecal constituents. In three patients receiving constant diets containing 76, 82 and 92 Gm. of fat daily, respectively, during successive six day periods of 1) control, 2) neomycin (12 Gm. per day orally), 3) neomycin plus ammonium chloride and citrate, 4) neomycin, 5) control, fecal excretion of the following was measured: total fat, free fatty acids, neutral fats, soaps, calcium, sodium, potassium and nitrogen.

Total fecal fat rose during neomycin administration in all three patients, the average percentage of daily intake in each period being: 1) 4 per cent, 2) 18 per cent, 3) 27 per cent, 4) 24 per cent, 5) 10 per cent. Soap excretion rose most markedly averaging eight times control. Free fatty acid excretion averaged six times control. Neutral fat excretion increased in only one period in one patient. Fecal calcium exceeded control excretion in all three of the neomycin periods in two patients but in only one period in the third. The average increase in fecal calcium was 200 mg. per day but the magnitude of increase did not correlate with the increase in fecal fat. Fecal potassium excretion rose by from 10 to 32 mEq. per day but fecal sodium was unchanged in two patients and increased by 5 to 15 mEq. per day in one patient. Fecal nitrogen increased consistently by from 1 to 1.9 Gm. per day. No deleterious clinical effects were noted and the changes reverted toward normal in all moieties with neomycin withdrawal.

The data indicate that oral neomycin produces a fecal excretory pattern similar to that commonly seen in sprue and which is accentuated by ammonium salts. This excretion results in significant fecal loss of calories and electrolytes in patients receiving neomycin. These observations may also suggest a technique for producing steatorrhea in the human.

The Febrile Response upon Injection of Bovine Albumin into Previously Sensitized Rabbits. RICHARD STUDLEY FARR, Pittsburgh, Pa. (Introduced by Wallace N. Jensen).

Fever is a manifestation of human allergy which can also be induced and measured in animals. An example is the fever thought by Stetson to be associated with the delayed type of hypersensitivity in tuberculous rabbits after an injection of nonpyrogenic products from tubercle bacilli. The present study was undertaken to determine whether rabbits could be sensitized to produce fever upon exposure to nonbacterial antigens.

Thirty-six rabbits were immunized with multiple intravenous injections of 10 to 25 mg. bovine serum albumin (BSA). Two to four weeks after the last injection, more than one-half of these rabbits responded with fever when challenged with 1 to 10 mg. BSA intravenously. The febrile response resembled that from bacterial endotoxin in that both exhibited: 1) a latent period between the injection and onset of fever; 2) a biphasic temperature curve lasting six hours; 3) development of tolerance after repeated daily injections; 4) restoration of a febrile response in tolerant rabbits by a tenfold increase in the dose injected; and 5) loss of tolerance following intravenous thorium dioxide.

The quantitative precipitin test and the ammonium sulfate method of measuring antigen-binding capacity revealed circulating antibody in all rabbits which responded with fever, while fever was never observed when antibody could not be detected. Conversely, however, fever did not always result when circulating antibody was present, and the correlation between fever response and total measurable antibody content was poor.

These experiments indicate, therefore, that hypersensitivity associated with fever is not restricted to the bacterial hypersensitivity of injection and provide further evidence that parenteral injections of nonbacterial antigens can induce fever similar to that resulting from infection.

The Pulmonary Collateral Blood Flow in Man. ALFRED P. FISHMAN,* GERARD M. TURINO, MARTIN BRANDFONBRENER, and AARON HIMMELSTEIN, New York, N.Y.

Anatomic observations have established that an extensive overgrowth of systemic arteries into the lung may accompany various types of heart and lung disease. The present study was designed to measure the rate of blood flow through such vessels, in particular the rate of collateral blood flow to the gas-exchanging surface of the lung ("effective"). For this purpose, the Fick principle was applied to three groups of subjects; in each group, the application was modified to conform with the anatomic abnormality. Thus, in the group of four subjects with congenital atresia of the pulmonary artery, the measurement of oxygen uptake and the oxygen content of arterial blood sufficed; in the group of two subjects with permanent occlusion of a major pulmonary artery, the combination of bronchospirometry, cardiac catheterization and arterial cannulation was employed; finally, in the group of six subjects with either acquired pulmonary disease or idiopathic clubbing of the digits, this combination was modified by the use of a balloon-tipped catheter to occlude transiently a pulmonary artery. Different rates of "effective" collateral blood flow were measured, ranging from zero in subjects with either carcinoma of the lung or recent occlusion of the left pulmonary artery, to approximately normal values for pulmonary blood flow in some subjects with congenital atresia of a pulmonary artery. Intermediate values, up to 1 liter per minute, were obtained in subjects with bronchiectasis, longstanding ligation of a pulmonary artery, and idiopathic clubbing of the digits.

Some Responses of the Pulmonary and Systemic Circulations of Man to Acute Alterations of the Alveolar Carbon Dioxide Tension. C. W. FRANK, A. JORDAN, F. KIEFHABER, and M. ZINN, New York, N. Y. (Introduced by Saul R. Korey).

Interest in a possible influence of the alveolar gas or arterial blood tension of carbon dioxide upon the pulmonary circulation stems from several theoretical and clinical considerations. The data to be presented concern the responses of 31 patients with rheumatic heart disease studied by means of right heart catheterization.

After a suitable control period during which the vascular pressures were recorded frequently and the cardiac output twice (Fick principle), an inspired gas containing 3 or 5 per cent carbon dioxide in air was administered and continued long enough to permit stabilization of the measurable parameters of ventilation and circulation (20 to 40 minutes), at which time the cardiac output measurement was repeated. The subsequent responses to another concentration of inspired carbon dioxide, a bout of voluntary hyperventilation breathing room air, and leg exercise (breathing air and then CO_2), were studied in various combinations.

In all 31 patients the inhalation of carbon dioxide resulted in an increase of the pulmonary artery mean pressure. The magnitude of this increase varied from 1 to 27 mm. Hg, with an average increase of 12 mm. Hg. Hyperventilation *per se* was shown not to be the cause of the rise. Changes in the cardiac output were inconstant, and did not correlate with the pressure rise. The "total resistance" to pulmonary outflow was increased above control in almost all subjects during the periods of respiratory acidosis.

Although the data so far available do not permit the clear identification of the mechanisms responsible for the increased hindrance to pulmonary arterial outflow, the response of several individual patients to suitably matched periods of exercise suggests that the "pulmonary vascular resistance" may have been increased by the respiratory acidosis.

The interplay of a variety of factors in the development of this pressor effect will be discussed.

The Effect of Smoking on Pulmonary Function in a Working Adult Population. WILLIAM FRANKLIN, Boston, Mass. (Introduced by Francis C. Lowell).

During a complete routine annual medical examination, 1,000 employees of a plant free of toxic or irritating dusts and fumes performed kymographic tracings of maximal forced expiration (E). They also answered a questionnaire dealing in detail with smoking habits and with respiratory symptoms and disease. In establishing standards for E it was found that measurement of the rate of flow between the point where 50 and 75 per cent of the vital capacity had been expired (E_{50-75}) was a reliable means of detecting early bronchial obstruction, and that it was less influenced by errors in performance than (E_{25-75}) or the volume expired in the first second.

In those between the ages of 40 and 65 years, unselected with respect to evidence of respiratory disease, the E_{50-75} was lower among those who smoked heavily than among those who smoked little or not at all. Among 105 male heavy smokers the mean E_{50-75} was $2,055 \pm 94$ ml. per second as compared with a mean of $2,520 \pm 102$ ml. per second for 103 males who smoked little or not at all. Among 36 female heavy smokers the mean was $1,575 \pm 130$ ml. per second as compared with a mean of $1,850 \pm 97$ ml. per second among 50 females who did not smoke. The p values for these differences were less than 0.01 for males and 0.05 for females.

These results indicate that heavy smoking can impair pulmonary function, that such effects are common among the population at large, that they frequently occur in the absence of any pulmonary abnormality of which the subject is aware, and that the abnormality does not represent a reaction peculiar to a few especially susceptible individuals. It is suggested that this abnormality is the forerunner of chronic obstructive pulmonary emphysema.

The Metabolism of Insulin by Human Placental Tissue. NORBERT FREINKEL * and CHARLES J. GOODNER, Boston, Mass.

In diabetics, requirements for insulin are markedly increased during pregnancy and acutely diminished in the immediate postpartum period. The possibility that placental degradation of insulin might contribute to this changing pattern prompted an inquiry into the metabolism of insulin by human placental tissue. Slices and homogenates of placenta were incubated with unlabeled and I^{131}-labeled crystalline beef insulin. In both cellular and cell-free preparations, proteolysis of the insulin could be demonstrated by the liberation of *a*) nonprotein nitrogen; *b*) free amino acids; and *c*) trichloracetic acid-soluble radioactive and ultraviolet-absorbing moieties. Loss of hypoglycemic potency was documented by bioassay. The placental system for the proteolytic degradation of insulin is thermolabile and may be inhibited by Zn^{++}, Cu^{++}, iodoacetate and *p*-chloromercuribenzoate. Activity of homogenates is enhanced in the presence of citrate buffers and reduced by dialysis. More than 80 per cent of the activity is localized within the soluble supernatant obtained by centrifugation at $100,000 \times G$. Analysis of the kinetics of insulin degradation revealed that the human placenta at term is at least as active as rat liver. No data are available for other human tissues.

The effects of extracellular factors upon this system were assessed. Insulin degradation by homogenates of normal placenta was studied during incubation with paired sets of maternal and cord sera obtained at delivery from normal and diabetic subjects. In seven normal pairs, insulin degradation was less in the presence of maternal than of cord serum. In four pairs from insulin-treated diabetics, both maternal and cord sera inhibited placental degradation of insulin. In the latter bloods, insulin-binding antibody was demonstrated by starch-block electrophoresis. Quantitative analyses of antibody titers were similar in each pair of sera, thus indicating free transplacental exchange of insulin-binding antibody.

The data support the suggestion that the human placenta may act as a major site of insulin degradation. Further studies are in progress to elucidate the possible role of this phenomenon in producing or modifying the abnormalities of the pregnant diabetic.

Effect of Acetazolamide (Diamox®) on Thyroid Function. J. L. GABRILOVE, New York, N. Y. (Introduced by L. J. Soffer).

The intravenous infusion of acetazolamide (Diamox®) into four euthyroid and two hyperthyroid subjects in a dosage of 125 mg. per kilo of body weight resulted in a decrease in the thyroidal uptake of radioactive iodine (I^{131}). Uptake was measured at 1, 2, 4, 6, and 24 hours following the oral administration of the tracer. The maximal depression obtained in the euthyroid group was 16, 27, 30, and 40 per cent of the control uptake and was generally noted in the 24 hour measurement. In the hyperthyroid group, the maximal decrease in uptake was observed in the six hour measurement and was 16 and 25 per cent of the control values. The effect on thyroid function is probably not secondary to the action of acetazolamide on the renal clearance of iodide since the administration of this agent to two patients with myxedema did not alter the urinary excretion of I^{131}. In these latter patients urine was collected at frequent intervals for a period of 50 hours.

Acetazolamide is a sulfonamide carbonic anhydrase inhibitor. In *in vitro* studies employing the colorimetric method of Wilbur and Anderson (J. biol. Chem. 1948 **176**, 147) carbonic anhydrase was demonstrated to be present in rat thyroid and the activity of the enzyme could be inhibited by adding acetazolamide to the test system. In other *in vitro* studies iodide was found to interfere with the action of purified carbonic anhydrase.

From these studies definitive proof cannot be adduced as to the importance of carbonic anhydrase in thyroid function. However, further investigations are in progress to test such a hypothesis.

Some Pathophysiological Aspects of Paroxysmal Nocturnal Hemoglobinuria. FRANK H. GARDNER,* EU-

GENE D. ROBIN, DAVID M. TRAVIS, DESMOND G. JULIAN, and CHARLES H. CRUMP, Boston, Mass.

The occurrence of enhanced hemoglobinuria during sleep has been described previously in paroxysmal nocturnal hemoglobinuria (PNH). It has been demonstrated *in vitro* that hemolysis may be increased by exposure of the abnormal erythrocyte to increased serum hydrogen ion concentration. It has been proposed that the respiratory acidosis of sleep is the major factor which accentuates hemolysis. The validity of this hypothesis has been studied in four patients with PNH.

Hourly measurements of plasma hemoglobin concentrations, arterial and venous blood pH, and breath to breath alveolar CO_2 tensions were determined during sleep and control intervals prior to and following the sleeping period. Two of the four patients showed a progressive rise in plasma hemoglobin during sleep. The time sequence of the increased hemoglobinuria did not correlate with respiratory acidosis. Two patients showed unvarying plasma hemoglobin levels during sleep, despite the development of respiratory acidosis. In one subject arterial and venous plasma hemoglobin levels were identical although there was a mean pH difference of 0.04 units. Two subjects inhaled 6 per cent CO_2 in air for 30 minutes. With this procedure a more profound respiratory acidosis developed than was observed during physiologic sleep. However, no significant further rise in plasma hemoglobin was obtained in the subsequent two hour period.

Nocturnal hemolysis in this disease is not related to the physiologic respiratory acidosis of sleep.

The Contrasting Ratios and Significance of the Tetrapyrryl (Bilirubinoid) and Dipyrryl ("Fuscin") Compounds of the Feces, in Certain Anemias. A. SIGRID GILBERTSEN, Minneapolis, Minn. (Introduced by C. J. Watson).

Earlier studies with P. T. Lowry, using N^{15} glycine, indicated that the fecal dipyrrylmethene or "fuscin" compounds are derived mainly from anabolic sequences on the pathway to heme rather than schism of tetrapyrrylbilirubinoids from destroyed hemoglobin.

The fecal "mesobilifuscin," partially purified by a modification of Siedel's method, has been compared in amount with urobilinogen in a group of normal subjects and in various anemias. The range of excretion of mesobilifuscin in normal human feces is 7 to 18 mg. per day and the urobilinogen/mesobilifuscin ratio (U/Mbf) is 8 to 14. In the presence of active hemolysis a disproportionate increase in fecal mesobilifuscin as compared with urobilinogen is often seen; hence the U/Mbf ratio is normal or low. This is also observed in cases of megaloblastic anemia. Remarkably low mesobilifuscin values are observed in cases of hyporegenerative or "refractory" anemia, even when the urobilinogen is normal or increased in amount, and in these the U/Mbf ratio is usually increased, often in marked degree. These results indicate that the magnitude of effective or attempted heme synthesis is more significant than hemoglobin destruction in determining the amount of mesobilifuscin in the excreta. Observations in certain other cases of anemia are also in accord with this concept.

Yet in some cases of anemia, especially those with rheumatoid disease, the amount of fecal mesobilifuscin has been small and the U/Mbf ratio high, despite an apparently normal or increased erythropoietic activity. This is as yet unexplained, but the possibility is considered that in these cases the immediate precursors of the "fuscin" compounds are more efficiently utilized.

The most consistent reduction of fecal mesobilifuscin and elevation of the U/Mbf ratio has been noted in cases of liver and biliary tract disease, probably due to diversion of mesobilifuscin to the urine, where marked increases are noted.

The Relationship of Serum Hexosamine Elevations to Immunological Reactions. ROBERT B. GILES, JR., MYRA MICHAEL, and JOE ED SMITH, Dallas, Texas. (Introduced by Elias Strauss).

Elevated serum hexosamine concentrations have been demonstrated in a group of diseases (collagen diseases, multiple myeloma, chronic infectious diseases) which are noteworthy for chronicity and tissue destruction, and, furthermore, which may be associated with immunologic disturbances. The elevated serum hexosamine concentrations have been attributed to: *a*) immunologic derangements; *b*) connective tissue destruction and/or proliferation of cells, particularly fibroblasts; *c*) destruction of leukocytes; and *d*) pyrogenic reactions. These experiments were undertaken to test these hypotheses and particularly to investigate further the role immunologic stimuli play in serum hexosamine alterations in the absence of tissue destruction.

Rabbits were sensitized to crystalline bovine plasma albumin (BPA), administered intravenously in 100 mg. doses at monthly intervals. Animals injected with saline served as controls. In order to dissociate serum hexosamine elevation from circulating antibody formation, on the one hand, and from leukopenia and hyperpyrexia, on the other, several animals were given 500 roentgens whole body radiation prior to antigenic stimulation, or were given *E. coli* endotoxin intravenously sufficient to cause hyperpyrexia and severe leukopenia. Serum hexosamine and precipitable antibodies to BPA were measured prior to and at frequent intervals after each antigenic stimulus.

These studies revealed: *a*) The initial injection of BPA did not elevate serum hexosamine concentration and elicited only minimal antibody response; *b*) Subsequent BPA injections caused serum hexosamine elevations four to six days following each antigenic stimulus, preceding or coinciding with a significant antibody rise; *c*) X-radiation prior to the challenging BPA injection suppressed both the antibody and hexosamine elevation; *d*) Endotoxin alone, sufficient to cause profound leukopenia and hyperpyrexia, followed by leukocytosis, was not associated with elevation of serum hexosamine.

These studies suggest that in the rabbit serum hexosamine elevations are related to immunologic responses occurring without apparent tissue destruction and do not appear to be related to severe leukopenia, leukocytosis or pyrogenic reactions.

The Site of Catabolism of Plasma Albumin. DAVID GITLIN,* JAMES R. KLINENBERG, and WALTER L. HUGHES, Upton, N. Y., and Boston, Mass.

While the organs or cells responsible for the synthesis of many plasma proteins have been established, the sites involved in the normal degradation of these proteins remains in question. Consequently, this study was undertaken to evaluate the relative importance of various organs in the catabolism of a major protein of plasma: albumin. Mouse and human serum albumins were labeled with I^{131} and given intravenously to mice. Behavior of both albumins was identical. The biological half-lives of albumin was determined by assay of radioactivity in the living whole mouse and by estimation of protein-bound radioactivity in pooled sera and in individual whole mice. The half-life of serum albumin was 0.7 days in normal mice; withdrawal of food increased the albumin half-life to 1.1 days. Sham operations did not significantly alter these values. Resection of 40 per cent of the liver markedly increased the albumin half-life to 1.7 days representing a 50 per cent decrease in the fractional rate of catabolism. Subsequently, albumin catabolism returned to normal as the liver regenerated. Removal of 15 per cent of the liver decreased albumin catabolism by 20 per cent. Bilateral nephrectomy or bilateral ureteral obstruction decreased albumin catabolism slightly, but resection of large and small intestine did not. Blockade of the reticuloendothelial system with thorotrast or India ink profoundly decreased albumin catabolism. The data indicate that the reticuloendothelial system is the major site of albumin catabolism and that the reticuloendothelial system of the liver plays the most prominent role.

Urinary Bacteriological Studies from Patients with Inlying Catheters. HARRY GLENCHUR, ROGER V. HAGLUND, WENDELL H. HALL,* and ROBERT H. WILCOX, Minneapolis, Minn.

Forty-three patients requiring an inlying catheter have been studied to determine the incidence of bacteriuria, the time required to acquire new species and their comparative resistance to antibiotics. Thirty-two patients acquired 55 strains of bacteria (over 5,000 per ml.); 17 of these patients had coincidental chemotherapy, as did 6 out of 11 acquiring no new strains. More resistant paracolons and fewer susceptible staphylococci were acquired with chemotherapy. New bacteria were found in one to three days. The acquired strains were resistant to the antibiotics used for prophylaxis.

Nine patients with protracted catheter drainage were given bladder lavages to determine the effect on their marked bactiuria. Lavage solutions included 2 per cent neomycin; crystalline penicillin G (1,000,000 units per L.) with streptomycin, chloramphenicol and neomycin (one gram each per L.); nitrofurazone (Furacin®) 0.012 per cent; acetate buffer (pH 4.4); and aqueous benzalkonium chloride 1:10,000. The solution was instilled in 50 ml. amounts b.i.d. through the catheter and left for 30 minutes. Quantitative urine cultures and antibiotic resistance tests were obtained weekly. The results were disappointing, as heavy bactiuria persisted. Sensitive bacterial species were eradicated, only to be replaced by more resistant bacteria or Candida species. The odor of the urine diminished.

Three patients without infection had a catheter inserted using neomycin unguent as the lubricant. Large numbers of Candida appeared within 7 to 14 days.

The findings indicate the potential danger of inlying urinary catheters.

Therapeutic Attempts with Cephalin Fraction of Bovine Brain in Thrombocytopenia and Classical Hemophilia. FRANCO GOBBI and MARIO STEFANINI,* Boston, Mass.

The protein-free fraction of bovine brain (Folch) was used *in vitro* and *in vivo* to correct the hemostatic defect of thrombocytopenia and hemophilia. *In vitro,* cephalin: *a*) shortened the clotting time and increased the prothrombin utilization of thrombocytopenic plasma at a lowest concentration of 1.7 mg. per cent; *b*) substituted for platelets in thromboplastin and thrombin generation tests; and *c*) at concentration of 50 mg. per cent corrected the prothrombin utilization in plasma of classical hemophilia; it was practically ineffective with PTC-deficient plasma. All five subfractions of cephalin exhibited platelet-like function, which was not, therefore, limited to phosphatidylethanolamine.

For use *in vivo,* cephalin was repeatedly washed with acetone and homogenized in saline. Aliquots were administered intravenously in 250 ml. saline into two patients with classical hemophilia and to seven with secondary thrombocytopenia. Results varied. Administration of 2.5 to 5 Gm. to thrombocytopenic patients induced: *a*) improvement of hemorrhagic manifestations; *b*) shortening of whole blood and of clotting time of recalcified plasma; *c*) normal utilization of prothrombin and labile factor (V) during coagulation; and *d*) improved generation of thrombin. Effects lasted 8 to 24 hours. Skin tests and repeated injections at short and long intervals failed to reveal sensitization or progressive reduction of therapeutic effects. An unexplained dissociation was seen after intravenous administration of 5 Gm. of cephalin to patients with classical hemophilia: *a*) the clotting time remained prolonged; *b*) the prothrombin utilization was greatly enhanced for at least eight hours, the effect being comparable to that of one unit of plasma.

It is concluded that: *a*) Cephalin at varying doses completely corrects the coagulation defect of thrombocytopenia and classical hemophilia *in vitro; b*) Cephalin of bovine origin is not antigenic and may be administered safely to humans. Significant, total or partial correction of the coagulation defect in classical hemophilia and

thrombocytopenia may thus be achieved *in vivo* with the use of a material of origin other than human.

Granulomatous Lesions—An Expression of the Hypersensitive State. MOSHE B. GOLDGRABER, Chicago, Ill. (Introduced by Joseph B. Kirsner).

The histologic study of chronic ulcerative colitis, based on surgical specimens and autopsy material, revealed granulomata with or without giant cells in a considerable number of cases. This finding, while a not uncommon feature of regional enteritis, has not been emphasized in ulcerative colitis. It has been suggested by Goddard that granulomatous inflammation is a "qualitative" characteristic of the hypersensitive state. The reproductibility of granulomas and giant cells, consequently, was investigated in sensitized and nonsensitized animals. Thirty guinea pigs were injected repeatedly into the subcutaneous tissue of the abdomen with crystalline egg albumin. After two weeks, 15 animals received four "eliciting" injections of the same antigen on four successive days; 15 received horse serum. Biopsies, taken on the third day after the last injection, demonstrated diffuse histiocytosis, proliferation of endothelial and perithelial cells in the test animals, at the site of the injections of egg albumin. Granulomas with giant cells were observed in 5 of 12 surviving animals given egg albumin, whereas only one of the control group developed diffuse histiocytosis and multinucleated cells. In additional experiments, granulomas with or without giant cells were noted in the colon of rabbits in serial biopsy studies of the Arthus and Shwartzman reactions 7 to 12 days after the induction of these reactions in the bowel wall. The granulomata in the subcutaneous and colonic sites appeared similar morphologically. It is suggested that granulomata with or without giant cells are an expression of the hypersensitive state.

Physiological Diagnosis of Disordered Coronary Circulation. RICHARD GORLIN,* NORMAN BRACHFELD, PIERRE BOPP, and COLIN MACLEOD, Boston Mass.

Objective physiological evaluation of the coronary circulation is needed in the diagnosis of coronary artery disease and evaluating therapy thereof. Such may be achieved by measuring coronary flow (CF) and vascular resistance (CVR) before and after induced vasodilatation.

Coronary venous catheterization was performed in 18 patients. Coronary flow per 100 Gm. left ventricular (LV) muscle was measured by the nitrous oxide technique at rest, and 5 to 12 minutes after 0.6 mg. sublingual nitroglycerin with simultaneous measurement of LV oxygen consumption, systemic arterial pressure, heart rate and indicator dilution output.

Resting coronary flow (66 ml. per 100 Gm. per minute) and oxygen consumption (8.3 ml.) increased an average of 63 per cent (110 and 13.6 ml., respectively) with a 42 per cent fall in CVR after nitroglycerin in 10 noncardiacs. LV work remained unchanged and efficiency decreased. In eight patients with angina pectoris, due either to coronary artery disease or aortic valvular disease, coronary flow and oxygen consumption (normal at rest) fell or was unchanged, while CVR remained fixed ($\pm$ 10 per cent). LV work and efficiency fell precipitously in these eight.

Failure of coronary flow to increase with nitroglycerin in this second group is believed to indicate prior maximal vasodilatation (in relation to either obstructed coronary arteries or increased oxygen work demands), and as such may serve as a physiologic test to detect coronary insufficiency.

This study suggests that nitroglycerin may not relieve ischemic pain by increasing coronary flow, but may do so by sharply decreasing cardiac work (even at the expense of efficiency) as suggested in 1867 by Brunton.

Micropuncture Study of the Osmolality of Renal Tubular Fluid in Potassium-Depleted Rats. CARL W. GOTTSCHALK,* MARGARET MYLLE, ROBERT W. WINTERS, and LOUIS G. WELT,* Chapel Hill, N. C.

Potassium-depleted rats are unable to elaborate as concentrated a urine as normal rats, and an anatomical lesion has been described in their collecting ducts. Previous micropuncture experiments in normal rats have demonstrated that the tubular fluid is made hypo-osmotic in the loop of Henle by extraction of solute, probably NaCl, in excess of water. In the presence of antidiuretic hormone, tubular fluid again becomes isosmotic by the end of the distal convolution and the hyperosmotic concentration occurs as it flows through the collecting ducts. The following experiments were designed to localize the site of the concentrating defect in potassium-depleted rats.

Tubular fluid was collected by micropuncture from 18 distal convolutions in 4 anesthetized, hydropenic, potassium-depleted rats. The osmolality of the tubular fluid and simultaneous urine and plasma was determined microcryoscopically. The site of micropuncture was established by subsequent microdissection. The osmolality of the distal fluid was similar to that of the normal group, despite the fact that the urine was less concentrated. The defect is thus localized to the terminal hyperosmotic concentrating process. It is not known whether the defect is the result of decreased permeability of the collecting ducts to water due to their anatomical lesions, or to some other factor. The transfer of water across the collecting duct epithelium may be due to its diffusion into the interstitium of the medulla made hyperosmotic by the reabsorption of NaCl in excess of water in the loops of Henle. The latter mechanism appeared normal in these rats since their early distal fluid was as hypo-osmotic as in controls, and they excreted as dilute a urine as controls when water-loaded.

Cardiovascular Dysfunction During Rickettsial Toxemia. SHELDON E. GREISMAN and CHARLES L. WISSEMAN, JR.,* Baltimore, Md.

Hypotension characteristic of typhus fevers is attributed generally to peripheral vascular damage and, more controversially, to myocarditis. Despite the usual temporal coincidence between vascular dysfunction and vascular

histological lesions, fulminant cases may succumb before classical tissue reactions develop, suggesting the possibility that vascular toxins may also participate. Determination of man's susceptibility to known rickettsial toxins has not been feasible. Yet, the remarkable vascular effects induced in susceptible animals by typhus toxins strongly attract consideration. This report contributes new information about vascular effects of murine typhus toxin.

Intravenous injection of murine typhus toxin into white rats in amounts lethal in about 150 minutes induces a characteristic, reproducible pattern of changes in the mescoappendiceal vascular bed. After about 30 minutes, large arterioles, small arterioles and precapillaries manifest constriction that gradually intensifies until death. Simultaneously, capillary blood flow diminishes progressively, finally leaving the capillary bed markedly ischemic. Hemoconcentration is generally present terminally. Nevertheless, sensitivity of terminal arterioles and precapillaries to topical epinephrine remains constant throughout. Systemic arterial blood pressure in *unanesthetized* animals remains essentially unchanged until near death but it falls sharply in *anesthetized* rats at about the time that arteriolar constriction and slowed blood flow first appear and before hemoconcentration occurs.

These observations suggest: *a*) Increased vascular permeability, previously established, develops in a remarkably specific manner that leaves unaltered vascular epinephrine reactivity and capacity to participate in reflex compensatory mechanisms. *b*) The VEM mechanism usually operative in acute blood loss does not function. *c*) Cardiac output, at least in anesthetized animals, diminishes appreciably early in the toxemia prior to, and initially independent of, reduction in total blood volume from hemoconcentration. These distinctive vascular changes differ significantly from those we have observed after acute hemorrhage and after lethal amounts of typhoid endotoxin.

In Vivo Demonstration of Splenic "Conditioning" in Hereditary Spherocytosis—Alterations in Osmotic and Mechanical Fragility Related to Erythrocyte Aging and Splenic Sequestration. ROBERT C. GRIGGS, RUSSELL WEISMAN, JR., and JOHN W. HARRIS,* Cleveland, Ohio.

The increased erythropoiesis of hereditary spherocytosis made it possible in two patients to label new red cells *in vivo* with Fe^{59}, and produce an identifiable group of tracer cells of known age.

When first delivered to the peripheral blood, the group of labeled cells was less fragile to osmotic or mechanical forces than the general population. The cell fragilities progressively increased over a period of 10 to 17 days, a proportion of these cells becoming even more abnormal than the general cell population. Counting radioactivity over body surfaces indicated progressive erythrocyte sequestration in the spleen. The cells showing the most marked increases in osmotic fragility disappeared from the peripheral circulation within a few days.

At splenectomy blood recovered from the spleen was labeled with chromium^{51} and returned to the patient. This blood obtained from the spleen contained a large proportion of cells with a marked increase in osmotic fragility; these disappeared from the peripheral circulation within 48 hours. The remaining erythrocytes survived normally.

These *in vivo* studies indicate that the spleen "conditioned" the susceptible erythrocyte over a period of several days resulting in progressive increases in osmotic and mechanical fragility relative to cell age. If returned to the peripheral circulation, such a cell probably survives less than 48 hours. In the splenectomized patient cells can no longer be sequestered and subjected to "conditioning." Accordingly, the survival time is normal even though the erythrocytes continue to show the defects typical of hereditary spherocytosis.

Effects of Calcium on Urine Concentrating Ability. JACOB GROSSMAN, MARTIN F. MINES, ARTHUR G. GOLDMAN, and MORRIS WOLFMAN, New York, N. Y. (Introduced by Louis Leiter).

Several diseases of diverse natures producing long-standing hypercalcemia and/or hypercalciuria often result in renal insufficiency manifested by impaired urine concentrating ability and polyuria. This observation led to the investigation of the acute effects of intravenous calcium adminstration on urine composition and concentration. Experiments were performed as follows: Isotonic lactated Ringer's solution containing inulin, PAH and vasopressin (Pitressin®) (0.5 to 2.0 mU per Kg. per hour) was infused at 1.0 to 1.5 ml. per minute throughout the procedure. When a constant flow of hypertonic urine was obtained, a second infusion containing isotonic calcium chloride or gluconate (0.75 to 1.5 mEq. per Kg. per 120 minutes) was begun. Serum and urine osmolarities and electrolyte concentrations and renal hemodynamics were measured.

Calcium administration invariably produced a gradual, progressive increase in urine flow to 6 to 17 ml. per minute despite the continued vasopressin. Moreover, during control studies without vasopressin in the same patients, similar maximal urine flows were achieved suggesting that calcium either inactivates circulating vasopressin (ADH) or renders the renal tubules insensitive. The diuresis was associated with a rise in renal hemodynamics and natriuresis. While these changes resemble those of osmotic diuresis, the fall in urinary osmolarity to hypotonic levels (positive free water clearance) indicates dilution in excess of solute demands.

The observed diuresis is not dependent simply upon total serum calcium concentration since the urine flow falls shortly whereas the serum calcium level decreases very gradually. The similar impairment of urine concentrating ability of potassium deficiency and calcium excess suggests that their known antagonism, perhaps due to effects on membrane permeability, extends to the kidney and is based upon their relative rather than absolute concentrations. However, in one study, the simultaneous administration of KCl (15 mEq. per hour) and calcium

gluconate (27 mEq. per hour) during vasopressin administration did not prevent the usual diuresis.

Erythropoietin in Fractionated and Unfractionated Plasma. CLIFFORD W. GURNEY, Chicago, Ill.

Erythropoietin, an erythrocyte stimulating hormone, has been demonstrated in normal plasma and its concentration is increased in the plasma of some anemic animals and human beings. In bioassay procedures usually employed, most of the protein has been removed from the plasma by heat-denaturation and precipitation. We have studied the loss of activity resulting from fractionation of plasma and have subjected whole as well as fractionated human plasma to bioassay for erythropoietin.

Male Sprague Dawley rats in which erythropoiesis has been depressed by hypophysectomy, acute starvation, or transfusion-induced polycythemia were used for bioassay, employing the 16 hour incorporation of Fe^{59} by erythrocytes following two injections of plasma or heat-denatured plasma extract. In all three assay preparations used, heat-denaturation was responsible for a striking loss of erythrocyte stimulating activity of anemic rat plasma. Therefore the erythrocyte stimulating property of unfractionated normal and anemic human plasma was compared to that of extracts of the same specimens prepared by boiling for 10 minutes. The average Fe^{59} incorporation produced by whole plasma from nine normal donors, when tested in 42 starved rats, was 5.7 per cent (standard error equals 0.39 per cent). This average value was reduced to 4.3 per cent (standard error equals 0.30 per cent) when the plasma was fractionated. In 12 of 24 anemic patients, whole plasma specimens increased the Fe^{59} uptake to levels in excess of 10 per cent. In every instance, loss of activity was produced by fractionation, and only three plasma specimens were clearly positive after extraction.

Although it is generally held that erythropoietin is heat stable, these results indicate noteworthy amounts of activity are lost by fractionation, and some negative results in the literature may be explained on this basis.

Erythrocytic Acetylcholinesterase Defect in Paroxysmal Nocturnal Hemoglobinuria. ROBERT C. HARTMANN, JOSEPH V. AUDITORE, and WILLIAM C. HOLLAND, Nashville, Tenn. (Introduced by John B. Youmans).

While much attention has been given to the study of the various plasma factors in the paroxysmal nocturnal hemoglobinuria (PNH) hemolytic system, relatively little investigation has been directed toward the PNH erythrocyte. It is believed that the stroma of the PNH red cell is defective although the nature of the abnormality is not settled.

Previous work in this labortary indicates that there is a close correlation between permeability and the activity of the acetylcholinesterase system in normal red cells. Therefore, the activity of this enzyme system in PNH cells was investigated. In four patients with PNH the red cell acetylcholinesterase activity was 25 to 80 per cent lower than in normal red cells.

Reticulocyte-rich erythrocyte portions (top layers of packed cells) showed much higher acetylcholinesterase activity than reticulocyte-poor portions (bottom layer of packed cells) in both normal and PNH subjects. The increase in enzyme activity was approximately proportional to the reticulocyte concentrations. Reticulocyte-rich portions of PNH cells contained higher concentrations (5 to 10 per cent) of reticulocytes than in the normal. Nevertheless, PNH reticulocyte-rich portions showed considerably less enzyme activity than normal reticulocyte-rich or -poor portions. It appears that this basic enzyme defect is present in both young as well as mature PNH cells. PNH reticulocyte-rich and -poor portions of red cells were equally susceptible to hemolysis in the Ham-Crosby test. The *serum* cholinesterase activity was also low in PNH.

On the Diagnosis of Functional Coarctation. HANS H. HECHT,* DALI J. PATEL, and RAMON L. LANGE, Salt Lake City, Utah.

In four subjects with coarctation of the aorta, two arterial catheters (1 mm. ext. diameter) were passed simultaneously through a brachial and femoral artery. This simple and harmless technique permitted exact localization of the coarcted segment *a*) from qualitative and quantitative pressure differences proximal and distal to the lesion, *b*) from wedging of one or both catheters into the coarctation or passing through the narrowed channel, *c*) localizing the site of the defect by measuring the length of the catheters from their insertion to the wedge position. The pressure gradients as well as the slopes of these central pulse curves were determined. Slopes measured 670 to 955 mm. Hg per second above, and around 140 mm. Hg per second below, the coarctation (normal range, 538 ± 86). The differences remain significant with changes in flow. Anatomical coarctation may occur without change in mean pressure or pulse pressure. Occasionally constriction may be shown by X-ray studies but without variation of either slope or pressure values. This suggests that the slope value is the most critical index of functional coarctation. Animal experiments carried out by causing mechanical aortic constriction in dogs, as well as hydraulic and electrical analogues, demonstrate that the anatomic constriction alone can account for many if not for all of the pressure phenomena of coarctation, and that the earliest and most consistent finding is change in slope of the pressure pulse curve below the lesion.

Direct Measurement of Cholesterol Absorption via the Thoracic Duct in Man. LEON HELLMAN,* E. L. FRAZELL, and R. S. ROSENFELD, New York, N. Y.

The intestinal absorption of cholesterol has been directly studied in man by cannulation of the left thoracic duct and complete collection of lymph following oral administration of cholesterol-4-C^{14} and acetate-2-H^3. This procedure permitted simultaneous observations concerning biochemical alterations and kinetic behavior of dietary cholesterol during the absorptive process as well as the

contribution to lymph of endogenously synthesized cholesterol.

From 4 to 27 per cent of the cholesterol-4-C^{14} was absorbed into lymph collected up to three days. Cholesterol absorption is a slow process, reaching a maximum in six hours, with significant quantities still being absorbed during the second day. Despite complete diversion of the lymph outflow of the left thoracic duct, a small quantity of C^{14} cholesterol still appeared in plasma. From this it was calculated that the fraction of the tracer dose absorbed through all other routes was less than one-fifth the amount which was absorbed through the left thoracic duct. In contrast, tritium-labeled biosynthetic cholesterol appeared in plasma in quantities and at rates usually observed in intact subjects indicating that acetate is absorbed through the portal system. The specific activities of lymph free and ester biosynthetic cholesterol were lower than simultaneous plasma samples. This is due to the expected dilution of plasma cholesterol, which serves as the source of the biosynthetic lymph cholesterol, by newly absorbed dietary cholesterol lacking the tritium label.

A minimum of 50 per cent of the C^{14} lymph cholesterol was in the ester form indicating that dietary cholesterol is esterified in the intestine during absorption. The plasma of *intact normo*cholesteremic subjects contains labeled free, but *no* labeled ester cholesterol during the first few hours following an oral tracer dose of cholesterol. However, labeled plasma ester cholesterol *is* present in identical studies in intact *hyper*cholesteremic subjects. It is therefore apparent that the ester cholesterol of lymph must be efficiently cleared from the circulation of *normo*cholesteremic subjects immediately following absorption and that either the esterification or clearance mechanism is defective in *hyper*cholesteremic subjects.

Effect of Ether Anesthesia on Human Skeletal Muscle Metabolism. DOROTHY H. HENNEMAN and LEROY D. VANDAM, Boston, Mass. (Introduced by Francis D. Moore).

Phosphocreatine, total creatine, and lactic acid concentrations have been measured in triplicate on nontraumatized paired biopsies of rectus abdominis muscle taken from women during 20 to 140 minutes of ether anesthesia for ovarian biopsy, hysterectomy, or cholecystectomy. The data were correlated with changes in blood pH and serum inorganic phosphorus. Twenty minutes after induction concentrations of phosphocreatine were 14 to 23 micromoles and total creatine 25 to 37 micromoles per gram of wet tissue; lactic acid was 0.12 to 0.84 micromoles per gram of wet tissue. During two hours of anesthesia, phosphocreatine fell to 8.5 to 10.8 micromoles per gram and total creatine to 11 to 20 micromoles per gram. Lactic acid concentrations showed no significant changes. Biopsies from anesthetized dogs without surgery showed comparable changes. Serum inorganic phosphorus increased as expected without any significant change in pH.

Ether anesthesia appears, therefore, to inhibit the synthesis of phosphocreatine without an associated increase in lactic acid. This may reflect a decrease in glycolysis. Studies during ether anesthesia in man have demonstrated abnormal elevations in blood glucose and serum inorganic phosphorus following administered glucose or epinephrine. In addition, there occurs resistance to the glucose and phosphorus-lowering effects of administered insulin. These abnormalities are not associated with abnormalities in lactic, pyruvic, or citric acid concentrations under the same conditions. Ether in some manner alters the transfer of glucose across the cell membrane. It is unknown whether this reflects a change in cellular permeability, insulin activity, or phosphorylating reactions associated with the transfer of glucose.

The Metabolic Defect Responsible for Uric Acid Renal Stone Formation. PHILIP H. HENNEMAN, STANLEY WALLACH, and ELEANOR F. DEMPSEY, Boston, Mass. (Introduced by A. P. Forbes).

Uric acid renal stones occur primarily in middle aged and elderly persons and especially in Jews and Italians. In a series of 22 patients with uric acid stones 5 had gout, 3 had chronic diarrhea and 14 were otherwise normal. In patients with the latter idiopathic variety of uric acid stones serum uric acid levels are nearly always normal and, more significantly, urinary uric acid excretion is not increased above normal (150 to 900 mg. per day) on unselected diets. Two constant biochemical peculiarities in patients with idiopathic uric acid stones are hypocalciuria and constantly very acid (pH 4.5 to 5.5) urine. Since normal human urine is supersaturated with uric acid at pH values less than 6.0 the cause of precipitation of uric acid in these patients is probably the constantly very acid urine.

Complete balance studies on neutral ash diets in two patients with idiopathic uric acid stones have shown persistently acid urines but have not shown excessive fecal base loss. In two uric acid stone patients the pCO_2 of urine collected under oil was not increased. Balance studies on neutral ash diets with measurement of all major urinary electrolytes in four idiopathic uric acid stone patients demonstrated an inverted ratio of ammonium to titratable acidity-minus-bicarbonate due to subnormal ammonia secretion. The administration of 140 mEq. of ammonium chloride daily for six days elicited less increase in urinary ammonium and a greater degree of systemic acidosis in two patients with idiopathic uric acid stones than it did in three normal subjects.

Thus it appears that most uric acid renal stones are the indirect consequence of an acquired isolated defect in renal tubular ammonia secretion.

Mechanism of Intrinsic Factor Action in the Isolated Rat Small Intestine. VICTOR HERBERT, New York, N. Y. (Introduced by Robert G. Bloch).

The effect of hog intrinsic factor concentrate (HIFC) on Co^{60}-labeled vitamin B_{12} uptake by sacs of everted rat small intestine was studied by methods previously applied

to a similar study with rat liver slices. Vitamin B_{12}-Co^{60} uptake was not enhanced when intestine sacs were incubated in a mixture of HIFC and vitamin B_{12}-Co^{60}. Up to fivefold enhancement was repeatedly observed when the sacs were incubated with HIFC, washed three times, and then incubated with vitamin B_{12}-Co^{60}. Using sequential incubation, it was demonstrated that maximum increase of mucosal vitamin B_{12}-Co^{60} uptake did not occur if calcium was replaced in the incubation medium by sodium. The uptake produced by HIFC was reversible to an appreciable degree by ethylenediaminetetraacetate. The similarity of these findings to those obtained with rat liver slices suggests the mechanism of HIFC action is identical in both systems.

That the increase in mucosal vitamin B_{12}-Co^{60} uptake after preincubation with HIFC is due to its intrinsic factor is suggested by the facts that: *a*) The increase is abolished by preheating the HIFC at 100° C. for 15 minutes. *b*) Performance of either incubation at pH 1.85 markedly reduced the effect of HIFC. *c*) 5, 6-Dimethylbenzimidazole, either alone or in combination with $CoCl_2$, does not affect vitamin B_{12}-Co^{60} binding in the system, as it does not affect vitamin B_{12} absorption *in vivo*.

It is postulated that intrinsic factor has the ability on one hand to bind vitamin B_{12} and on the other to attach to liver or intestine receptors in the presence of calcium. Possession of these two properties simultaneously may be unique to intrinsic factor.

Pathways of Ribose Synthesis in Normal and Pentosuric Human Subjects. HOWARD H. HIATT, Boston, Mass. (Introduced by H. L. Blumgart).

Ribose synthesis from hexose via the pentose phosphate pathway has been observed in several mammalian systems. Recently, an additional mechanism for ribose biosynthesis has been postulated as a result of the demonstration in animal tissues of the following reactions: D-glucuronic acid → L-gulonic acid → L-xylulose → xylitol → D-xylulose → D-xylulose 5-phosphate → D-ribulose 5-phosphate → D-ribose 5-phosphate. A block in this pathway would explain the excretion of L-xylulose by persons with the genetic disorder, essential pentosuria.

We have studied ribose biosynthesis in man with a trapping technique suggested by Tabor and Hayaishi's observation that imidazoleacetic acid (IAA) riboside appears in the urine following IAA administration. Patients given 1,500 micromoles of IAA and glucose-2-C^{14} excreted as much as 570 micromoles of riboside ribose containing 0.05 per cent of administered C^{14}. Fifty per cent of the C^{14} in the ribose molecule appeared in carbon 1, and 39 per cent in carbon 2, consistent with hexose conversion to ribose via the pentose phosphate pathway. Administration of IAA and D-glucuronolactone-U-C^{14} to a subject with normal carbohydrate metabolism was followed by the excretion of ribose containing 0.06 per cent of the administered C^{14}. In striking contrast, a pentosuric subject given IAA and glucuronolactone-C^{14} excreted a comparable quantity of ribose, which was virtually free of C^{14}, and a large amount of L-xylulose-C^{14}.

From our observations we conclude that human subjects with normal carbohydrate metabolism may synthesize ribose by at least three mechanisms: *1*) the oxidative loss of carbon 1 of hexose phosphate; *2*) the nonoxidative series of reactions catalyzed by transketolase and transaldolase; and *3*) conversion of glucuronic acid to ribose via L-xylulose. Our data provide strong support for the postulated block in the third pathway in essential pentosuria. Evidence from human studies and from experiments with rats given IAA and glucose-3,4-C^{14} will be presented to indicate that the first two mechanisms are quantitatively most significant in ribose biosynthesis.

The Mode of Action of Chlorothiazide and Mercurial Diuretics as Antihypertensive Agents. WILLIAM HOLLANDER and ARAM V. CHOBANIAN, Boston, Mass. (Introduced by Robert Wilkins).

The antihypertensive action of chlorothiazide and mercurial diuretics was studied in 20 hypertensive and 20 normotensive subjects. Oral and intravenous chlorothiazide or parenteral mercurial diuretics significantly reduced the blood pressure in 14 of 20 hypertensive subjects but did not alter blood pressure in the normotensive controls. Changes in weight, sodium excretion, and creatinine clearance were comparable in both groups. Body sodium (Na^{24} space) and extracellular fluid volume ($S^{35}O_4$ space) were not significantly altered in 12 hypertensive subjects after prolonged oral chlorothiazide therapy, although significant reductions in body potassium (K^{42} space) occurred.

Sixteen metabolic balance studies revealed: *1*) The antihypertensive effect of chlorothiazide or mercurial diuretics was greater than that of a 9 mEq. sodium diet alone. *2*) Net sodium losses as small as 50 mEq. were found during the antihypertensive effect of chlorothiazide or of mercurial diuretics, but were usually (as were net potassium losses) as large as 150 to 250 mEq. *3*) Upon simultaneously withdrawing the diuretics and reducing the dietary intake of sodium to 9 mEq. daily, the antihypertensive effect and cumulative negative sodium balance persisted until sodium intake was increased, but cumulative potassium balance reverted to pretreatment levels. *4*) Increase in dietary sodium intake to as much as 225 mEq. daily did not block the antihypertensive action of chlorothiazide. *5*) Addition of 9-α-fluorohydrocortisone to the chlorothiazide regimen restored sodium balance to control levels without blocking the antihypertensive effects. *6*) Addition of the steroidal antagonist SC-8109 (Searle) [19-nor analog of 3-(3-oxo-17β-hydroxy-4-androsten-17α-yl), propionic acid γ-lactone] produced further loses in body sodium and increased the antihypertensive effect of chlorothiazide.

In conclusion, chlorothiazide and mercurial diuretics appear to have a selective antihypertensive action which is not necessarily dependent on, but which may be potentiated by a reduction in body sodium. Maintenance of a reduced body sodium may prolong the antihypertensive effects of these diuretics.

Cross Resistance to Cerebral Typhoid Infection and Influenza Virus Neurotoxicity. EDWARD W. HOOK and ROBERT R. WAGNER,* Baltimore, Md.

Salmonella typhosa inoculated intracerebrally in mice produces an acute purulent meningo-encephalitis and death in 1 to 10 days. Large doses of non-neurotropic influenza virus injected intracerebrally also cause an inflammatory reaction of the brain and meninges, characteristic tonic convulsions and death in 48 to 72 hours. Salmonellae multiply rapidly to high titer in brain tissue, whereas proliferation of influenza virus at this site is extremely limited. Despite marked differences in pathogenicity and the nature of the infecting organisms, resistance to both infectious agents can be induced in strikingly similar fashion. Animals injected intracerebrally with typhoid vaccine 24 hours previously resist challenge with either *S. typhosa* or influenza virus to about the same degree. Injection of subtoxic doses of influenza virus also protects mice against virus neurotoxicity although pretreatment with virus has only a minimal effect on cerebral typhoid infection.

The most pronounced refractory state is produced by purified bacterial endotoxins; lipopolysaccharides prepared from *Escherichia coli* or *S. equiabortus* markedly inhibit bacterial multiplication in brain and protect mice against 400 LD_{50} of *S. typhosa*. The route of inoculation is also important. Ten micrograms of *E. coli* lipopolysaccharide injected intraperitoneally in mice produces only a slight increase in resistance to intracerebral challenge, whereas 0.01 microgram of lipopolysaccharide given intracerebrally affords effective protection against both cerebral typhoid infection and influenza virus neurotoxicity.

Resistance to either typhoid infection or virus toxicity develops in a few hours, is maximal at 24 to 48 hours, and gradually disappears in about a week. The similarities in time of onset, duration and degree of host resistance to two such different types of infection are additional evidence for a general phenomenon of acquired resistance unrelated to specific immunity.

Adequacy of Renal Oxygenation in Essential Hypertension. WILLIAM E. HUCKABEE, Boston, Mass. (Introduced by Chester S. Keefer).

The question whether anoxia is present, in the sense that rate of O_2 supply is insufficient to meet requirements, cannot be answered by measuring the blood flow to or even the mean P_{O_2} in a tissue. To determine whether renal anoxia in this sense is present in patients with essential hypertension, rates of anaerobic metabolism (AMR) of their kidneys were compared with those of normal subjects. From analyses of 24 hour urines for lactate, pyruvate (corrected for ketoglutarate) and creatinine, the urinary excretion of excess lactate, XL, was estimated (lactate unaccounted for by the normal $DPNH_2$/DPN ratio). Both groups excreted widely varying quantities of total lactate (U/P ratio < 1.0) and pyruvate (U/P ratio > 1.0), but excretion of calculated XL (the result of anaerobiosis) was relatively constant in any one individual during two to three years. The mean rate of excretion of XL in 22 hypertensive patients without pyelonephritis was significantly greater than in normal subjects (109 mEq. per Gm. creatinine per day, $p < 0.01$). It was not affected by exercise, infusion of pyruvate or lactate, or chronic administration or restriction of salt, nor by reduction of blood pressure during reserpine therapy. However, AMR of the kidney could be increased by reducing blood pressure with ganglionic blocking drugs or, in dogs, by constricting the renal artery. Renal AMR was proportional to the mean starting blood pressure (untreated) ($r = 0.66$), but was independent of current blood pressure. It was concluded that the kidneys of these hypertensive patients were abnormally dependent upon anaerobic metabolism because of relative inadequacy of O_2 supply to the urine-producing cells (those capable of altering tubular filtrate composition with respect to lactate), since the only mechanism for altering lactate concentrations is the intracellular lactic dehydrogenase system, which also necessarily reflects the $DPNH_2$/DPN ratio of the cells.

Pre-Albumin, a New Thyroxine-Binding Protein of Human Plasma: Its Isolation and Physiologic Activity in Normal and Abnormal States. SIDNEY H. INGBAR,* Boston, Mass.

During electrophoresis in veronal buffer, physiologic concentrations of thyroxine migrate in association with moieties in the interalpha area, termed thyroxine-binding protein (TBP). In contrast, in a new electrophoretic system to be described, endogenously and exogenously labeled thyroxine migrate both with TBP and with protein moving approximately 20 per cent faster than albumin at pH 8.6 (pre-albumin). Comparative studies in the two systems indicate that failure to observe thyroxine-binding by pre-albumin in veronal is due either to trailing or pre-albumin or to protein interactions in this buffer. In the new system, physiologic concentrations of thyroxine distribute almost equally between TBP and pre-albumin. Additionally, in contradistinction to findings during conventional electrophoresis in veronal, total thyroxine bound to TBP reaches a plateau as the concentration of thyroxine is increased. Thus, absolute binding capacities of both TBP and pre-albumin can be assessed. In normal sera, the total thyroxine-binding capacity of TBP approximates 25 μg. per 100 ml. of serum, whereas that of pre-albumin is approximately 100 μg. per 100 ml.

Thyroxine-binding capacity of TBP is normal in patients with untreated Graves' disease and increased in pregnant women at term. However, in both groups, binding capacity of pre-albumin is markedly decreased. Findings in other abnormal states are being assessed.

Pre-albumin seems distinct from TBP for the following reasons: *a*) In pathologic sera, the absolute thyroxine-binding capacity varies independently of that of TBP. *b*) At low carrier concentrations, TBP binds triiodothyronine, but pre-albumin does not. *c*) TBP is present in Cohn Fractions IV-7 and IV-8 of human serum, whereas

pre-albumin is not. *d*) Treatment of proteins with neuraminidase alters the isoelectric point of TBP, but not of pre-albumin.

Pre-albumin of exceedingly high thyroxine-binding potency has been isolated in an electrophoretically and ultracentrifugally homogeneous state. Its sedimentation and electrophoretic characteristics, as well as its amino acid and carbohydrate contents, have been ascertained and will be described.

The Demonstration of Bilirubin Sulfate in Bile. KURT J. ISSELBACHER, Boston, Mass. (Introduced by Mandel E. Cohen).

It is now apparent that the metabolism of bilirubin by the liver results in the formation of a water-soluble derivative which is excreted into the bile and gives a "direct" Van der Bergh reaction. Previous investigators have identified this pigment as the glucuronide conjugate of bilirubin.

Since the hepatic conjugation of other alcoholic and phenolic compounds, such as corticosteroids and estrogens, involves the formation of sulfate as well as glucuronide derivatives, it seemed reasonable that an ethereal sulfate conjugate of bilirubin at its hydroxyl group might also occur. Such a derivative would be water soluble, but, in contrast to the alkali-labile glucuronide ester, the ethereal sulfate derivative of bilirubin would be relatively alkali-stable. Previous observations that 10 to 15 per cent of the "direct-reacting" pigment of human bile was alkali-stable gave additional support for this hypothesis.

Bile was collected from rats with bile fistulas and from the gall bladder of cats following the intraperitoneal injection of S^{35}-labeled inorganic sulfate. After treatment with *p*-diazobenzenesulfonic acid, the resulting azo-pigments were purified and separated from bile salts by extraction with heptane-butanol and by adsorption of the pigments on zinc hydroxide gel at pH 7 to 8. Paper chromatography of the purified azo-pigments in two solvent systems and subsequent autoradiography consistently revealed the presence of radioactivity in the "direct-reacting" azo-pigment band. The eluted labeled material was stable to alkali at 25° C. and 37° C. but was readily hydrolyzed in 1 N HCl at 100° C. with the liberation of inorganic sulfate. Labeled taurine was not identified as a product of this hydrolysis. The azo-derivative of synthetic bilirubin sulfate was prepared and shown to have the same chromatographic properties as the labeled azo-pigment in the bile.

While bilirubin glucuronide probably represents the major constituent of the "direct-reacting" bile pigment, the present studies indicate that other conjugates, such as bilirubin sulfate, also occur.

Comparison of the Effectiveness of Transfusions of Fresh and Lyophilized Platelets in Controlling Bleeding Due to Thrombocytopenia. DUDLEY P. JACKSON,* DALE K. SORENSEN, EUGENE P. CRONKITE,* and VICTOR P. BOND, Upton, N. Y., and Baltimore, Md.

Enumeration of red blood cells in thoracic duct lymph provides a semiquantitative measure of the degree of bleeding in animals rendered thrombocytopenic by whole body irradiation. This method was employed to evaluate the hemostatic effect of platelets lyophilized by the method of Klein and his associates.

Seven dogs were rendered thrombocytopenic by the administration of 500 to 550 r whole body irradiation. After thrombocytopenia developed, large numbers of red blood cells were observed in thoracic duct lymph. Lyophilized dog platelets were infused in amounts calculated to increase the recipient's platelet level by approximately 200,000 per cu. mm. However, following infusions, the recipient's circulating platelets did not increase. The number of red blood cells in the lymph did not decrease significantly in any of the seven dogs after administration of the lyophilized platelet preparations.

Four of the dogs subsequently received infusions of freshly prepared platelet suspensions. Circulating platelet levels increased, and there was a dramatic decrease in the number of red blood cells in the lymph. In a representative experiment, the mean output of red blood cells in the lymph before infusion was 373 million red blood cells per minute. Following infusion of lyophilized platelets, the mean output was 466 million per minute. Following infusion of a comparable number of fresh platelets, the mean output decreased by approximately 95 per cent from 466 million per minute to a level of 17 million per minute.

Thus, lyophilized platelets did not circulate in thrombocytopenic recipients and did not effect hemostasis. Infusions of fresh platelets increased circulating platelet levels and had a dramatic hemostatic effect.

Common Cold Neutralization by Human Immune Globulin. GEORGE GEE JACKSON,* HARRY F. DOWLING, and TRUMAN O. ANDERSON, Chicago, Ill.

It is generally believed that infection with the common cold does not produce immunity to reinfection. It is not even known whether such an infection elicits a specific antibody response in the host and if so whether the antibody is protective. In the present investigations the common cold was transmitted to volunteers by means of filtered infectious nasal secretions. Pooled human immune globulin was tested for its capacity to neutralize the infectivity of two secretions which produced an acute infectious coryza after appreciably different incubation periods. Human albumin and buffered salt solution also were tested for neutralizing activity. Among 65 volunteers who were challenged with an infectious secretion suspended in salt solutions, 52 per cent developed a common cold. When the secretion was suspended in albumin solution, 12.0 Gm. per 100 ml., 50 per cent of 38 volunteers had an experimental cold. The use of human immune globulin, 8.0 Gm. per 100 ml., reduced the number of experimental colds to 10 per cent of 59 volunteers. During the time of the experiments "spontaneous" colds were observed in 15 per cent of 61 volunteers who were challenged with a control solution. The data are highly

significant and showed that the globulin fraction of human serum contains potent, heat labile substances capable of neutralizing infectious agents of the common cold in nasal secretions. The implication is that this is antibody elicited by previous natural infections. Studies on the presence of serum globulin in nasal secretions will be presented. The findings suggest that the susceptibility of the nasal epithelium to infection with common cold agents may be independent of circulating antibody.

Sequestration of Reticulocytes and of Abnormal Red Cells by Filtration at Low Pressures. JAMES H. JANDL,* Boston, Mass.

Reticulocytes from animals of several species possessed properties similar to antibody-coated red cells; they were selectively agglutinated by Coombs sera and by agents which induce rouleaux-formation. After severe anoxia from bleeding or phenylhydrazine injection, autoagglutination of reticulocytes in native plasma was observed. In animals reticulocyte levels of spleen pulp blood exceeded those of peripheral blood, as observed by Berendes, despite the absence of splenic erythropoiesis. Sequestration of reticulocytes was particularly striking during reticulocytoses, and was not observed in other organs.

In order to understand why reticulocytes and sensitized red cells are trapped in the normal spleen, while agglutinated red cells are sequestered in the liver, conditions were examined affecting the passage of red cells through Millipore filters with a pore size of 5.5 microns. Sicklemic red cells, at low oxygen tensions, and spherocytes were readily separated from normal red cells. Similarly, reticulocytes and antibody-coated red cells suspended in plasma fortified with polyvinylpyrrolidone or fibrinogen were selectively trapped at low perfusion pressures; higher pressures (above 8 to 10 mm. Hg) caused the cell clumps to dissociate, permitting passage of these cells. Red cells directly agglutinated by "complete" antibodies penetrated the filter only under still higher perfusion pressures. The ability of red cell rouleaux or agglutinates to penetrate filters was a function of the force required to dissociate these aggregates.

Thus, 1) immature red cells resemble antibody-coated red cells in their agglutinability and filterability *in vitro* and in their susceptibility to splenic sequestration *in vivo;* 2) splenic sequestration of reticulocytes may explain certain apparent suppressive effects on erythropoiesis of "hypersplenism"; and 3) the sites of sequestration of immature, abnormal, or agglutinated red cells may be determined largely by the pore sizes and perfusion pressures of different vascular filter-beds.

The Lysis of Artificially Induced Intravascular Clots in Man by Intravenous Infusions of Streptokinase. ALAN J. JOHNSON * and W. Ross MCCARTY, New York, N. Y.

The fibrinolytic effect of streptokinase, as first demonstrated by Tillett, has been firmly established. The present studies were undertaken to extend these observations and to demonstrate that intravascular clot lysis may be produced by the intravenous infusion of purified streptokinase (SK), in patients, under optimum biochemical conditions.

Blood clots 5 to 20 centimeters long were produced in peripheral veins of volunteers. Twenty-four or 48 hours later, SK was infused intravenously into a contralateral extremity. The presence of the clot was determined by inspection, palpation, and X-ray venograms taken before, during, and after the infusions.

Biochemical studies on each patient's plasma prior to the infusion made it possible to determine the SK required to neutralize the SK antibody and inhibitor, *in vivo*. Therefore, additional infused SK was free to be effective in an active fibrinolytic system. Further studies were performed during the infusion to maintain an appropriate balance of plasmin, plasminogen, plasmin-activator, SK and prothrombin.

In 11 instances, SK was infused in amounts calculated to obtain optimal clot lysis, *in vivo*. Eight clots, 24 hours old, lysed within 20 hours, and 3 clots, 48 hours old, lysed in 33 hours. No clot reformation occurred. Embolic complications have not been observed.

In addition, SK was deliberately infused in amounts smaller and larger than the calculated optimum. Clot lysis failed in seven instances when smaller than optimal amounts of SK were infused. Circulating plasmin was present in all seven without detectable free SK. Variable clot lysis occurred in six instances when larger than optimal amounts of SK were infused. Incomplete lysis, or clot reformation occurred in five of these, presumably due to a deficiency of circulating plasminogen. One clot lysed completely without reforming.

The results demonstrate that SK will produce intravascular clot lysis when infused intravenously into patients under defined biochemical conditions.

Genetic and Intrauterine Influences on Steroid Hormone Production in Man. ATTALLAH KAPPAS and T. F. GALLAGHER, New York, N. Y. (Introduced by Allan T. Kenyon).

Isolation, characterization and quantitative estimation of individual steroid metabolites during several experimental periods in identical triplets have demonstrated significant heritable and prenatal environmental influences on steroid hormone production. Urinary ketosteroids comprising amounts of androsterone, etiocholanolone, dehydroisoandrosterone, 11-hydroxyandrosterone, 11-hydroxyetiocholanolone and 11-ketoetiocholanolone form patterns that are reproducible and constant for any individual over long periods. These patterns differ quantitatively from subject to subject. Individual differences likewise appear during stimulation with adrenocorticotrophic hormone (ACTH).

The steroid patterns of adult, normal male dichorionic monozygotic triplets were examined in detail during control and ACTH periods. In two triplets these patterns were qualitatively and quantitatively virtually identical before, during and after ACTH administration. This concordance, in view of the highly individual nature of

these patterns, is impressive evidence of an important heritable influence on steroid hormone metabolism. The third triplet, however, produced significantly smaller amounts of these metabolites during all periods. This finding is interpreted as evidence for the existence of an important environmental influence by means of which steroid metabolism had been modified. The intrauterine focus of this influence is strongly suggested by the small birth weight and slower growth of this triplet compared with his brothers. Related physiological differences in monochorionic twins have been attributed to demonstrated vascular asymmetry and unequal distribution of the mutual fetal circulation to one partner of the pair. The dichorionic character of the triplet membranes makes it evident that a fetal situation comparable to that seen in such twins existed for two of the brothers. It can be presumed, therefore, that the triplet with these divergent steroid patterns was one of this fetal pair and that his differences in production and metabolism of steroids represent a physiologic aftermath of relatively deficient fetal circulation.

Asymptomatic Bacteriuria and Pyelonephritis of Pregnancy. E. H. KASS,* A. L. KAITZ, R. COTRAN, W. L. CLOUGH, and R. L. NICHOLS, Boston, Mass.

Relatively high rates of occurrence of asymptomatic bacteriuria have been found in various population groups. Among these, pregnant women were found to have more than 10^5 bacteria per ml. of urine in 11 per cent of specimens obtained at delivery. Further studies have shown the following:

1. Clean voided specimens may be used for the detection of bacteriuria by quantitation of the bacterial flora. The agreement with results obtained using catheterized specimens was greater than 95 per cent. The use of catheterized specimens for quantitative bacteriologic study of urine is rarely necessary.

2. Asymptomatic bacteriuria was found in 10 per cent of about 4,000 patients making *first* prenatal visits. Bacteriuria was not related to the duration of pregnancy and was found as early as the second month.

3. Untreated bacteriuria persisted throughout pregnancy and about 40 per cent of patients with bacteriuria at the first prenatal visit developed symptomatic pyelonephritis during the last trimester or in the immediate postpartum period.

4. When asymptomatic bacteriuria was suppressed in alternate patients by continuous antibacterial treatment, pyelonephritis did not occur.

5. Reliable differentiation of patients with and without bacteriuria on the basis of history, physical findings, or pyuria could not be made.

6. Significant residual urines were not found in the bladders of bacteriuric patients, using a method which eliminates catheterization.

7. The bacteriologic flora of the vaginal secretion in patients with bacteriuria was not significantly different from the flora of patients without bacteriuria; coliform organisms were rarely found in either case.

The results suggest that asymptomatic bacteriuria and pyelonephritis are pathogenetically related, and that detection and management of bacteriuria may eliminate pyelonephritis of pregnancy. The demonstration of bacteriuria early in pregnancy suggests that bacteriuria may persist in many young women between pregnancies or that a metabolic disturbance occurring early in pregnancy predisposes to the development of bacteriuria.

Anergy and the Response to Homografts in Hodgkin's Disease. W. D. KELLY, R. A. GOOD,* and R. L. VARCO, Minneapolis, Minn.

At present the best evidence indicates that homograft rejection in man is an immune response which in many details closely resembles the phenomenon termed delayed bacterial hypersensitivity. A recent report that Hodgkin's disease is associated with a high incidence of anergy to common bacterial and viral antigens applied as skin tests would appear to offer an opportunity to evaluate the role of delayed hypersensitivity in homograft rejection in man. The present report concerns such a study. At first, 22 patients with Hodgkin's disease and 207 patients not having lymphoma or terminal cancer were skin tested with the following antigens: 1) Schick control (diphtheria toxoid), 2) streptokinase-streptodornase, 3) mumps virus, 4) mumps control solution, 5) trichophytin audouini, 6) candida albicans, and 7) P.P.D., intermediate strength. The incidence of positive reactions was recorded by observation at 24 and 48 hours. The results show a high incidence of anergy in the Hodgkin's disease group in contrast to the control group.

Ten of the patients with Hodgkin's disease have had full-thickness skin homografts carried out. The grafts which were tattooed to aid in identification were followed by frequent observation for periods ranging from three to eight months. The results indicate a distinctly altered pattern of reaction varying from essentially complete acceptance of the graft to complete rejection. In every instance, except one, the rejection time was prolonged when compared to rejection times for skin homografts observed in immunologically normal persons. The predominant response observed is a slow, prolonged disintegration of the graft by repeated episodes of blistering, exudation with crust formation and partial sloughing.

These results confirm the high incidence of anergy in Hodgkin's disease and report a new finding of altered skin homograft rejection in these patients.

Stimulation of Amino Acid Penetration into Mammalian Skeletal Muscle by Insulin. DAVID M. KIPNIS and MATTHEW M. NOALL, Saint Louis, Mo. (Introduced by Robert E. Shank).

Insulin stimulates the incorporation of amino acids into proteins of skeletal muscle. To test whether this response is secondary to an accelerated rate of amino acid transport or to a direct stimulation of protein synthesis, a nonmetabolizable amino acid analogue, α-aminoisobutyric acid (AIB), has been used to study amino acid penetration in skeletal muscle.

Control studies indicated that AIB-1-C^{14} was neither incorporated into the protein or lipid of rat diaphragm or gastrocnemius nor oxidized to CO_2. Total tissue radioactivity was extracted with 0.008 M acetic acid and when chromatographed migrated in one spot corresponding with authentic AIB.

Rates of intracellular penetration were determined *in vitro* by incubating "intact" rat diaphragm preparations under conditions where the medium concentration of AIB (3.2×10^{-5} M) remained constant. AIB penetration was twice as rapid in diaphragms of rats fasted for 72 hours as in those of nonfasted animals. Insulin increases the rate of AIB penetration 300 to 500 per cent in fasted and nonfasted preparations in the presence or absence of glucose. Glucose (0.01 M) decreases the rate of AIB penetration 30 per cent in the presence or absence of insulin. In these experiments, AIB concentration gradients (intracellular content/extracellular content) of two to three were established. The rate of attainment of a steady-state distribution of AIB between plasma and muscle water and the final concentration gradients established in nephrectomized normal and diabetic animals were measured following the intravenous injection of 1 μM of AIB-1-C^{14}. The results of these *in vivo* studies were similar to those obtained with the *in vitro* technique.

These results indicate that current concepts of insulin action must be broadened to include the stimulation of both sugar and amino acid penetration. This suggests that impairment of amino acid penetration may contribute to the disturbed metabolism of diabetes mellitus.

The Effect of Parathyroid Extract (PTE) on the Renal Clearance of Diffusible Calcium. CHARLES R. KLEEMAN, ROBERT E. ROCKNEY, and MORTON H. MAXWELL, Los Angeles, Calif. (Introduced by Samuel H. Bassett).

In unpublished observations, the authors have noted that: 1) Hypoparathyroid subjects of recent origin, not on vitamin D, may have normal or elevated calcium excretion while *hypocalcemic*, and, after parathyroid extract administration, urinary calcium may fall while serum calcium rises. 2) Hyperparathyroids with hypercalcemia but normal renal function may have normal calcium excretion. 3) In normal subjects on high calcium diets, chronic PTE administration may cause hypercalcemia without further hypercalciuria. All the above suggest that parathyroid hormone may specifically decrease the renal *clearance* of calcium.

To clarify this point, paired studies of the renal clearance of diffusible calcium were determined in normal subjects during a four hour infusion of 1 Gm. of calcium ion ($CaCl_2$) with and without simultaneous administration of PTE. Serum diffusible calcium was obtained by the Lavietes anaerobic ultrafiltration technique.

The serum diffusible calcium averaged 59 per cent, and this magnitude was not affected by the PTE or calcium infusion. The pH of the serum and ultrafiltrate was constant (7.3 to 7.5) during the ultrafiltration. The basal clearance of diffusible calcium with PTE was 2.0 ml. per minute—without PTE, 4.5 ml. per minute. During the infusion the diffusible calcium clearance rose (mean peak, 12 ml. per minute), but, for any given filtered load of calcium, the clearance was always *less with* PTE ($C_{Diff.\,Ca.}$ PTE $\div$ $C_{Diff.\,Ca.} = 0.5$ to 0.6).

PTE inhibited the serum phosphorus rise associated with calcium loading by increasing the renal clearance of phosphorus. The injection of PTE into the renal artery of one dog caused a decrease in the calcium clearance on the homolateral side.

Studies are in progress to compare beef PTE with human PTE prepared in this laboratory.

It is concluded that PTE specifically *decreases* the renal clearance of calcium in humans, confirming the rat data of Talmage [Proc. Soc. exp. Biol. (N. Y.) 1955, **88**, 600.]. PTE (hormone?) maintains serum calcium by *three* mechanisms: a) it "liberates" bone calcium, b) *increases* renal phosphorus clearance, and c) *decreases* renal calcium clearance.

Immediate Wheal Hypersensitivity. I. The Diphtheria System as a Model in Studying the Nature of Reagins. WILLIAM J. KUHNS, Pittsburgh, Pa. (Introduced by Frank J. Dixon).

Although production of allergic reagins seems to be related to the genesis of immediate wheal allergies, little is known about their nature except that they are ordinarily classified as antibodies. The ability of reagins to sensitize skin characteristically refers to their persistence in skin tissues or mucous membrances, so that allergic antigens introduced later cause immediate wheal reactions. Passive transfer methods for detecting reagins are based upon these properties. However, for technical reasons, it was previously impossible to determine exact times of survival of these components in skin. Similarly, it has been difficult to exclude the possibility that skin persisting antibody in addition to other serum components collectively comprise reagin and are required for wheal production. If one antibody apart from other serum components can be calibrated in human skin, it may be possible, using appropriate skin testing methods, to determine whether: 1) only antibody is able to exhibit the biological properties of reagin, and 2) residence of antibody in skin differs appreciably from persistence in circulating blood.

Antibody which is also antitoxin may be measured using sensitive toxin neutralization techniques. Thus, rabbit skin tests were employed by us to accurately measure diphtheria antitoxin in immune sera from human subjects. It was found that when calibrated sera were passively transformed into Schick positive subjects, some antitoxins present in amounts equivalent to the Schick test dose could convert local skin areas to the Schick negative state for seven days or more. Toxic reactions caused by Schick reagent at control sites were used for comparison with test areas. Variations in this property, and associations with other immunological characteristics of antitoxin will be discussed.

Persistence of Antibiotics in Blood of Patients with Acute Tubular Necrosis and Uremia. CALVIN M. KUNIN and SEARLE REES, Boston, Mass. (Introduced by Maxwell Finland).

The "half-life" of antibiotics in serum was determined after single intravenous infusions, or after cessation of previous antibiotic therapy by assaying the serum concentrations of the antibiotics at intervals. Values were determined in normal subjects and in patients with acute tubular necrosis during oliguric or diuretic phases. The antibiotics studied were penicillin, streptomycin, tetracycline and erythromycin.

The half-life of penicillin G (crystalline sodium salt) after intravenous administration averaged 30 minutes in two normal subjects; it was 7 to 10 hours in two oliguric individuals with a urinary output less than 400 ml. per day. During the diuretic phase, the half-life in three patients ranged from 3.5 to 6.5 hours.

The half-life of tetracycline hydrochloride in four oliguric patients ranged from 60 to 120 hours. In one patient, the value was 60 hours after diuresis began and later, as diuresis progressed, it was 14 hours. In normal controls the values were 7 to 8 hours.

Streptomycin was given intramuscularly in two oliguric patients. In one of them the concentration in the serum remained unchanged over a period of 3 days and in the other the level fell to one-half its maximum in 19 days.

The half-life of erythromycin in one oliguric patient was found to be about 5 hours when the urine output was less than 400 ml. per day. Normal values were 1.5 and 1.1 hours.

These data are considered to be of importance in providing a rational approach to antibiotic therapy during acute tubular necrosis and uremia and in further defining the role of the kidney in their excretion. Further studies are underway on the persistence of these and other antibiotics in the blood of patients with chronic renal disease, during hemodialysis with the artificial kidney and on the *in vitro* decay of these drugs.

Dietary Fats and Blood Lipids. PETER T. KUO and JOHN C. CARSON, Philadelphia, Pa. (Introduced by Calvin F. Kay).

Similar dietary programs have yielded divergent effects upon plasma lipids as reported by different investigators. That some apparent discrepancies are based upon factors other than quantitative or qualitative changes in dietary fat is indicated by this investigation. In 18 patients receiving a 20 Gm. fat per day diet continuously for 10 to 54 months, the mean cholesterol, phospholipid, and triglyceride fell from 356, 320, and 302 mg. per cent, respectively, to 266, 256, and 146 mg. per cent, respectively. Ten of these patients then received a supplement of 27 to 54 Gm. per day of soya-bean oil for 6 to 18 weeks. This did not significantly alter the lipid levels. Four patients were placed upon a 3 to 12 week program of isocaloric regular, "fat-free," and 45 to 70 per cent corn oil formula diets in various sequences. Lipids were regularly and comparably lowered by either a "fat-free" or corn oil program when preceded by a regular diet. However, a "fat-free" program preceded by a corn oil program resulted in a rise in lipid levels in two of the patients. In one patient on a "fat-free" diet preceded by a regular diet, fasting triglyceride was stable and low for nine weeks, then abruptly doubled. Lipid determinations every two hours on this patient showed two peaks, one at 8 a.m., when fasting levels are usually determined, and a 2 a.m. trough. When a corn oil program was substituted, a single plateau was sustained throughout the afternoon and evening, followed by a decline that reached bottom at 8 a.m. This gives the impression, from observation of fasting levels alone, that on corn oil feedings triglyceride levels are depressed. These studies indicate that fasting lipid levels may be a misleading index of dietary effects and that responses may be profoundly influenced by the nutritional program of the preceding control period.

Studies on the Genetics of Leukocyte Antigens. PARVIZ LALEZARI and THEODORE H. SPAET,* New York, N. Y.

Leukoagglutinins were studied by the technique of Dausset. Sera positive for leukoagglutinins were obtained from 30 patients who had received from 2 to 700 transfusions. These sera were specific for leukocytes in that no mixed agglutination with erythrocytes occurred, and no erythrocyte antibodies were demonstrable. No false positive leukoagglutination was seen in a large number of normal sera; and spontaneous leukoagglutination was not encountered. Sera were tested only with leukocytes from blood with compatible red cells.

The abnormal sera were tested with leukocytes obtained from six pairs of identical twins. In each case twin leukocytes showed identical agglutination response patterns, indicating similar antigenic composition. The patterns differed among the different pairs of twins.

Three of the abnormal sera were tested against the leukocytes of the patients' own family members. Two of these sera gave negative results with a high percentage of the leukocytes from relatives, although most leukocytes from nonrelated subjects were agglutinated. The third serum agglutinated leukocytes from all family members tested.

The abnormal sera were tested against leukocytes from a large panel of normal and unrelated subjects. None of the sera was positive for the entire panel of leukocytes; and none of the leukocyte suspensions was agglutinated by all of the abnormal sera. All subjects had a different pattern of response to the various sera; and no two sera behaved identically to the panel of leukocytes.

The data indicate that there is a multiplicity of leukocyte antigens, and that these are probably inherited according to Mendelian laws.

Seven Year Follow-up of One Type of Prolonged Intermittent Steroid Therapy in Nephrosis. KURT LANGE, EDWARD WASSERMAN, LAWRENCE B. SLOBODY, and EUGENE J. WENK, New York, N. Y. (Introduced by A. F. Coburn).

Forty-six cases, 35 children and 11 adults, with the nephrotic syndrome ("pure" nephrosis and the nephrotic stage of glomerulonephritis) were treated with the following regimen. There was an initial course of adrenocorticotrophic hormone (ACTH), 100 to 200 units per day, given for 10 to 21 days and after complete diuresis had occurred, maintenance therapy consisting of 300 to 400 mg. of cortisone per day for three successive days out of each week. This maintenance therapy was carried out for 12 to 18 months. The average time of observation for this group after onset of maintenance therapy was 43.8 months. Every case coming under observation since 1951 was treated with this regimen. Twenty additional cases (12 children and 8 adults) are still under therapy or are not under observation for a minimum of 20 months as yet. They were not included in the statistics. None of the latter group, however, died, and only one is edematous at present.

According to the life table statistical method, the mortality expected in the group on maintenance therapy (on the basis of the mortality occurring in a similar group not treated with steroids or treated with steroids only when edematous) would have been 12.8 deaths during the period of observation. There has been one death in our group on maintenance therapy. Tested by the chi-square method, the possibility of this distribution occurring by chance was found to be less than one in a thousand ($\chi^2 = 10.87$, $p < 0.001$).

Of the group of 25 patients in whom this type of therapy was started within the first six months of their disease, 20 are at present free of proteinuria and 5 have a 1 plus proteinuria. Of the group of 20 patients in whom this therapy was started more than six months after onset of the disease, only 5 have no proteinuria, while 14 have a 1 plus proteinuria and one has a 3 plus proteinuria and he is moderately uremic.

Thus *early* treatment with ACTH to induce complete diuresis followed by maintenance therapy with steroids seems indicated.

In four cases resistant to repeated courses of intramuscular ACTH, complete diuresis was achieved by the intravenous infusion of 30 to 40 units of ACTH, over an eight hour period each day for 10 to 12 days.

Uric Acid Riboside Phosphorylase in Human Tissues—Inhibition by Colchicine, and Other Properties. LEONARD LASTER and ALBERTA BLAIR, Bethesda, Md. (Introduced by J. E. Rall).

Uric acid (UA), although generally regarded as only an end-product of purine metabolism in man, does, however, appear to be subject to active transport and catabolism in human tissues. Since uric acid riboside (UAR) has been detected in human erythrocytes, and many purine interconversions involve their riboside or riboside forms, a study of the metabolism of UAR in mammalian tissues was undertaken.

Accordingly, an enzyme was purified one hundredfold from dog intestine that catalyzes the phosphorolysis of UAR as follows:

$$\text{UAR} + \text{Inorganic phosphate} \rightleftharpoons \text{UA} + \text{Ribose-1-phosphate} \quad (1)$$

It (URA-P'ase) occurs in human spleen, liver, kidney, stomach and ileum, as well as in organs of other mammals. Human spleen UAR-P'ase was purified five- to tenfold and its properties were studied. Dog, and quite probably human, UAR-P'ase appear to be distinct from nucleoside phosphorylase (assayed with inosine), an enzyme that catalyzes the phosphorolysis of other purine ribosides. Colchicine is an inhibitor of UAR-P'ase but does not inhibit nucleoside phosphorylase activity under similar experimental conditions.

In equal concentrations, colchicine, phenylbutazone and a phenylsulfoxy derivative of phenylbutazone, G-28315 (1,2-diphenyl-3,5-dioxo-4-(β-phenylsulfoxy ethyl)-pyrazolidine), inhibited dog UAR-P'ase 45, 30, and 6 per cent, respectively, a relationship that correlates interestingly with their therapeutic effectiveness in acute gouty arthritis. Similar concentrations of probenecid and pyrazine monocarboxylamide, compounds affecting primarily renal disposition of urate, did not inhibit. Thiouracil and propylthiouracil were more potent inhibitors of human UAR-P'ase than was colchicine.

The findings in this study raise the question of whether previously unrecognized pathways of metabolism related to UA, such as reaction (*1*), are involved in the hereditary defect of purine metabolism in gout and in the pathogenesis and therapy of acute gouty arthritis. Clinical extension of these investigations would seem profitable for insight into human disorders of purine metabolism.

Reappraisal of Renal Hemoglobin Excretion: The Differential Transport of Free and Protein-Bound Hemoglobin. WILLOUGHBY LATHEM,* Pittsburgh, Pa.

The assumption that hemoglobin circulating in plasma exists altogether in a free, unbound state and freely traverses the glomerular membrane has provided the conceptual basis for studies of renal hemoglobin transport. The demonstration of hemoglobin binding by plasma protein(s) and the circulation of both free (unbound) and protein-bound hemoglobin have rendered this assumption invalid. In the present study the characteristics of hemoglobin excretion were reexamined, employing methods which permitted differentiation between the renal transport of free and protein-bound hemoglobin.

Hemoglobin was administered intravenously to normal subjects in multiple injections, raising progressively (three hour period) the plasma hemoglobin concentration to 200 to 300 mg. per cent. Urine and plasma were obtained for hemoglobin analysis following each injection, allowing 30 minutes for equilibration. The concentrations of free and protein-bound hemoglobin (PBH) were determined by paper electrophoresis, measuring the light absorption of the paper following staining with benzidine-hydrogen peroxide. Hemoglobinuria did not occur until the hemoglobin-binding capacity of plasma protein(s) was exceeded (124 ± 22 mg. per cent) and free hemoglobin

appeared in plasma. From the relationship between the changing plasma concentration and urinary excretory rate of free hemoglobin ($\Delta UV/\Delta P$), the glomerular clearance (C^{G}_{Hb}) of free hemoglobin was calculated and averaged 5.0 ± 1.1 (range, 3.9 to 6.8) ml. per minute. The tubular reabsorptive rate (T_{mHb}) of free hemoglobin was calculated as G^{G}_{Hb} times P_{Hb} U_{Hb} V and averaged 1.4 ± 1.0 (range, 0.0 to 2.8) mg. per minute. Methemalbumin, when present in plasma, was not excreted.

This study reveals: *a*) Hemoglobinuria does not occur until the binding capacity of plasma protein(s) is exceeded; *b*) The "renal threshold" is determined largely by this capacity; *c*) Free hemoglobin was excreted but PBH was not, indicating either absence of filtration or complete reabsorption of PBH; *d*) The excretory rate of free hemoglobin is determined largely by glomerular permeability, tubular reabsorption being small; *e*) Both permeability and reabsorption are less than previously estimated.

Observations on Hepatic Metabolism of Oxytetracycline in Man. CARROLL M. LEEVY, MYRA R. ZINKE, WOO YOON CHEY, JUNIUS SNELL, and ARTHUR ENGLISH, Jersey City and Maywood, N. J. (Introduced by Harold Jeghers).

Hepatic metabolism of nonradioactive and randomly labeled C^{14} oxytetracycline has been investigated in five normal subjects and four patients with moderately advanced Laennec's cirrhosis. Hepatic uptake and turnover of this antibiotic, and their effects on ammonium and oxygen metabolism over a four to six hour period, were studied utilizing the technique of hepatic vein catheterization. Two hundred and fifty milligrams of tagged oxytetracycline (specific activity about 0.1 microcuries per milligram) was given orally, or 100 milligrams of the nonradioactive antibiotic given parenterally. Antibiotic levels were determined from collected aliquots of intestinal juice, bile, urine and serum by microbiological and liquid scintillation radiochemical analyses. These levels were determined from percutaneous liver biopsies obtained one and six hours after oral administration of 250 milligrams of labeled oxytetracycline.

It has been previously demonstrated that radioactive oxytetracycline and tetracycline are concentrated in the liver of mice (Snell, Garkuscha and Allen). The present studies show that oxytetracycline also accumulates in the human liver. Initial serum levels of the antibiotic apparently result from lymphatic absorption as detection of oxytetracycline in hepatic venous blood is delayed; subsequent concentrations in peripheral blood appear to depend upon that released from hepatic stores. The uptake and egress of oxytetracycline were decreased in subjects with cirrhosis and correlated with estimated hepatic blood flow and biochemical function tests. There was a reduction in serum ammonium after either oral or parenteral administration of the antibiotic. This effect could not be wholly attributed to intestinal activity of oxytetracycline; following its parenteral use there was a decrease in hepatic venous ammonium before significant quantities of the antibiotic reached the intestine.

These data indicate that the liver serves as a storage center for oxytetracycline in man; intrahepatic metabolism of this antibiotic contributes to both its bacteriostatic action and effects on ammonium metabolism.

The Dependence of Plasma Potassium Concentration on Plasma Osmolarity, Blood pH, and Body Composition. J. LEIBMAN, L. W. BIRKENFELD, and I. S. EDELMAN,* San Francisco, Calif.

Plasma potassium concentration (K_p) may be influenced by body potassium content and blood pH. Theoretically, K_p might also vary with intracellular osmolarity, and instances of inverse correlations between K_p and plasma sodium concentration (Na_p) have been noted. An integrated account of the dependence of K_p on these factors, however, has not been achieved, nor have others been excluded. The present study is based on the proposal that the steady state concentration gradient of potassium across cell membranes is a function of body potassium, intracellular osmolarity and the partition of hydrogen ions in accordance with the Gibbs-Donnan effect. This concept was evaluated by simultaneous measurement of K_p, Na_p, arterial pH, total exchangeable potassium (K_e) and total body water (T.B.W.) in 30 patients with acid-base disturbances or abnormalities of K_p. Lean subjects were selected, and the ratio of K_e to "dry" body weight (body weight minus T.B.W.) was used as a measure of the potassium content of the lean body mass. Analysis of the data derived from these measurements revealed: a modest correlation between K_p and arterial hydrogen ion concentration ($[H^+]$) ($r = 0.47$); a similar limited correlation between K_p and K_e/"dry" body weight ($r = 0.41$); a somewhat higher correlation between K_p and the product $[H^+]$ times K_e/"dry" body weight ($r = 0.60$); a negligible correlation between K_p and Na_p ($r = -0.15$); and a significant but incomplete correlation between K_p and the ratio $[H^+]$ times K_e/"dry" body weight divided by Na_p ($r = 0.65$). Logarithmic coordinates did not modify these correlations. These results indicate a direct influence of K_e and extracellular pH on K_p and only an occasional relation to extracellular osmolarity. More strikingly, however, they demonstrate a hiatus in knowledge of some of the important parameters which control K_p in man.

Effects of Reduced Glomerular Filtration on Urine Concentration in the Presence of Antidiuretic Hormone. NORMAN G. LEVINSKY, DOUGLAS G. DAVIDSON, and ROBERT W. BERLINER,* Bethesda, Md.

Dogs in which the glomerular filtration rate (GFR) can be decreased reversibly in one kidney and urine collected separately from each kidney have been used to study the effects of reduced GFR on the elaboration of hypertonic urine. When the GFR of one kidney is lowered 10 to 25 per cent in dehydrated dogs infused with vasopressin, the concentration of the urine from that kid-

ney regularly becomes up to 40 per cent greater than that from the control kidney. Increases in urine osmolality are regularly noted even when the control concentration is as high as 1,800 to 2,000 mOsm. per Kg. H_2O. This indicates that the maximum urine concentrations found at very low osmolar excretions under ordinary conditions do not represent the maximum U/P ratio ("osmotic ceiling") which the kidney can achieve. When the reduction of GFR is 30 per cent or more, urine osmolality consistently falls markedly. In this circumstance analysis of renal tissue shows that urea concentration always and sodium (chloride) concentration usually is reduced in the medulla of the kidney whose GFR has been lowered. When urea does not contribute significantly to the urine osmolality and increased amounts of sodium salts reach the loop of Henle (mannitol diuresis), reductions of GFR of up to 70 per cent do not cause significant falls in urine concentration.

These data are consistent with the hypothesis, to be presented, that urine hypertonicity is developed by equilibration of fluid in the collecting ducts with hypertonic medullary interstitial fluid and that the medullary interstitial fluid is made hypertonic to plasma by deposition of sodium salts from water-impermeable Henle loops. Accumulation of urea in the same region, considered the result of passive diffusion from urea-permeable collecting ducts, makes possible the achievement of considerably higher osmolalities than could be developed in response to the interstitial sodium salts alone.

Effect of a Reduction in Filtered Load on Solute and Water Excretion in Hydropenic Man. MARVIN F. LEVITT, MARSHALL S. LEVY, and DEMETRA POLIMEROS, New York, N. Y. (Introduced by Alexander B. Gutman).

In eight normal subjects and four with advanced renal failure, the glomerular filtration rate (GFR) was reduced by venous obstruction of both lower extremities and/or the administration of a ganglionic blocking agent. For 18 hours prior to the study the patients were deprived of all fluid. During a two hour control and a comparable hypotensive period, throughout which intravenous vasopressin (Pitressin®) was administered at a rate of 200 milliunits per hour, renal clearances, urine osmolality, and the rates of solute and water excretion were measured.

In the normal subjects GFR was reduced to 60 per cent of control values with a concomitant fall in urine flow. The average urinary osmolality decreased 90 mOsm. per Kg. H_2O or 12 per cent from control levels, and the urea concentration fell 38 mOsm per Kg. H_2O or 20 per cent. The concentration of salt dropped markedly, the rate of chloride excretion falling to 10 per cent of control values. The average urinary potassium concentration rose 33 per cent producing a much smaller decrease in the rate of potassium excretion.

In the patients with renal failure the changes in GFR, chloride, water, and potassium excretion were similar to those described above. However, in these subjects the fall in GFR was associated with a rise in urea concentration of 40 mOsm. per Kg. H_2O and an increase in urine osmolality of 10 per cent.

An increase in urine urea and total solute concentration, as observed in the uremics, is expected when the quantity of isosmotic fluid reaching the concentrating segment is reduced. In the normals, the expected increment was obscured by a coincident diluting effect. The net dilution seems best explained by the continued back diffusion of urea at a site distal to that segment of the tubule where solute free water is extracted and maximum urinary concentration effected.

The Probable Identity of Glass Factor with Hageman Factor. JESSICA H. LEWIS* and WILLIAM R. MERCHANT, Pittsburgh, Pa.

Blood in contact with glass clots more rapidly than when in contact with a nonwettable surface such as silicone. This increased coaguability has been ascribed to activation of one or more of the various plasma coagulation factors or to removal of an inhibitor.

This glass-activated accelerator effect, herein called glass factor, may be demonstrated by mixing a small amount of glass treated material with normal silicone plasma and observing its effect on the recalcification time in a silicone tube. Plasma from patients suffering from deficiencies of AHF, PTC, proaccelerin, Stuart Factor and Factor VII shows normal glass factor. Normal plasma adsorbed with barium sulfate to remove prothrombin, proconvertin and PTC also shows normal glass factor content. Plasma from a patient with Hageman factor deficiency was devoid of this glass factor and a stored-frozen sample from a PTA deficiency had only minimal accelerating action.

When normal plasma was fractionated by continuous filter paper curtain electrophoresis some of the 32 fractions obtained (primarily in the γ area) showed the ability to accelerate the clotting of normal silicone plasma. The distribution of this "glass factor" corresponded almost exactly to that of the Hageman factor. On the other hand, when Hageman factor deficient plasma was so fractionated, neither Hageman factor nor glass factor activities were found in any of the fractions. The fractions from the Hageman deficient plasma were also assayed for anti-Hageman and antiglass factors, but no such activities could be detected. It is possible that an inhibitor could have been removed during the fractionation procedure.

A Circulating Antibody Directed Against Penicillin. ALLYN B. LEY, AMOS CAHAN, and KLAUS MAYER, New York, N. Y. (Introduced by Rulon W. Rawson).

It has been found that human erythrocytes incubated with high concentrations of penicillin (over 1 mg. per ml.) and subsequently washed and suspended in saline become agglutinable on exposure to the sera of certain individuals. All penicillin derivatives thus far tested, including synthetic penicillin, sensitize erythrocytes to react

with these sera. The inactivation of penicillin by penicillinase completely inhibits the sensitization.

The agglutinating factor in these sera resides in the gamma globulin fraction and retains its potency after heating at 56° C. for two hours. The prior addition of penicillin to the sera inhibits the agglutination of previously "penicillinized" erythrocytes.

Among 413 hospital patients tested, 41 (9.9 per cent) showed the presence of the agglutinin in their sera. Among 1,893 blood donors, tested by a relatively insensitive technique, 4 (0.2 per cent) gave positive reactions. All positive reactors about whom information was available had received or were receiving penicillin at the time the test was done. Skin and conjunctival tests with penicillin were negative in those individuals tested. In a group of 21 patients who gave a history of clinical penicillin sensitivity, 7 (33 per cent) showed the agglutinin in their sera.

Penicillin added to these sera *in vitro* exerted as great an antimicrobial effect as when added to control sera.

Erythrocytes exposed to high concentrations of penicillin (about 50 mg. per ml.) rapidly disappeared after transfusion into a patient whose serum contained the agglutinin, whereas they disappeared at an approximately normal rate in a normal control. No evidence has been found, however, that comparable hemolysis occurs when penicillin is administered in the usual manner.

It is concluded that this agglutinin is an antibody specifically arising as a consequence of penicillin exposure. The clinical significance of the antibody remains to be determined.

Modification of Adrenal Steroid Patterns in Man by Means of an Inhibitor of 11 Beta-Hydroxylase. GRANT W. LIDDLE,* DONALD ISLAND, HERSCHEL ESTEP, and GORDON M. TOMKINS, Nashville, Tenn., and Bethesda, Md.

Chart and co-workers demonstrated that 2-methyl-1,2-bis(3-pyridyl)-1-propanone (SU-4885) decreases adrenal vein 17-hydroxycorticoids of dogs. The present investigation indicates that SU-4885 induces in man biochemical changes resembling those of hypertensive congenital adrenal hyperplasia, that this may be due to specific inhibition of 11 β-hydroxylation, and that this phenomenon can be turned to advantage in evaluating pituitary function.

Although in four surgical patients SU-4885 caused abrupt *decreases* in adrenal vein hydrocortisone, in nonstressed subjects SU-4885, after several hours, caused marked *increases* in blood and urinary 17-hydroxycorticoids and urinary 17-ketosteroids. Despite high 17-hydroxycorticoid levels, no depression of circulating eosinophils or other evidence of hydrocortisone excess occurred.

It was postulated that SU-4885 might be a specific inhibitor of 11 β-hydroxylation. As such it would inhibit secretion of hydrocortisone, induce a compensatory increase in adrenocorticotrophic hormone (ACTH) secretion, and thereby cause a late rise in 11-desoxycorticosteroids such as "compound S." Chemically this would resemble the situation described by Eberlein and Bongiovanni in hypertensive congenital adrenal hyperplasia. The following evidence supports this postulate: *1*) SU-4885 has no effect on steroid levels in Addisonian patients maintained on exogenous hydrocortisone. *2*) SU-4885 does not cause an increase in steroid levels in normal subjects treated with hydrocortisone to preclude a "compensatory" rise in ACTH secretion. *3*) The 17-hydroxycorticoid which becomes elevated following SU-4885 has the chromatographic and chromogenic properties of the 11-desoxysteroid "compound S" rather than hydrocortisone. *4*) SU-4885 inhibits *in vitro* activity of a partially purified 11 β-hydroxylase derived from bovine adrenals.

Since the late rise in steroid levels is mediated by increased secretion of ACTH, SU-4885 has been used to test pituitary "reserve." Patients with pituitary tumors or with previously irradiated pituitary glands have failed to show increased steroid levels following SU-4885, suggesting that pituitary "reserve" was limited even though steroid levels were adequate under nonstressful conditions and even though responsiveness to exogenous ACTH was not lost.

Diverse Distribution of Red Cells and Plasma Albumin in Anatomical Regions of the Kidney. LAWRENCE S. LILIENFIELD, NIELS A. LASSEN, and JOHN C. ROSE, Washington, D. C. (Introduced by Edward D. Freis).

Red cells labeled with Cr^{51} and albumin labeled with I^{131} were injected simultaneously intravenously in eight dogs. Four renal pedicles were ligated after three minutes and 12 at the end of one hour. Blood pressure remained at normal levels throughout the procedure. Both kidneys were removed, frozen quickly, and sliced with a band saw. Various anatomic sections were removed, weighed, and counted with a well-type scintillation counter. Recounting after three weeks permitted measurements of each isotope separately. Tissue radioactivities were compared to radioactivity of arterial blood.

Red cells occupied 5.5 ml. per 100 Gm. outer cortex and 6.6 ml. per 100 Gm. of inner cortex. The anatomically highly vascular outer medulla contained 11.4 ml. red cells per 100 Gm. while the inner medulla contained 6.6 ml. The greatest variability was found in the papillae. In many animals the papillae contained less than 0.1 ml. red cells per 100 Gm. The average papillary red cell content was 3.7 ml. per 100 Gm.

One hour volumes of distribution of albumin were 23.5, 32.5 and 37.6 ml. per 100 Gm. of cortex, medulla and papilla, respectively. Papillary albumin was 85 per cent equilibrated in three minutes. All quantitative data were verified by radioautography.

It is concluded that: *1*) These data contradict the red cell concentration-plasma skimming theory of autoregulation, which predicts a higher red cell concentration in outer than inner cortex. *2*) The papillary red cell content is highly variable but on a comparative basis the renal papilla is extraordinarily deficient in red cells. *3*) Amounts and rate of albumin accumulation indicate that renal plasma albumin circulates extravascularly (a source of error in total plasma volume measurements). *4*) The

large amount of albumin in the papilla suggests that some is concentrated above that of plasma and might therefore be involved in a mechanism for the concentration of urine.

The Hemopoietic Effect of Batyl Alcohol. JAMES W. LINMAN, Chicago, Ill. (Introduced by Frank H. Bethell).

Observations previously reported by us indicate that there are at least two plasma stimulatory factors concerned with control of erythropoiesis. One accelerates erythroblastic cellular division and is thermostable, ether-soluble, and most likely a lipid. The other factor, which is relatively thermolabile, insoluble in ether, and probably protein in nature, augments hemoglobin synthesis. Batyl alcohol, the monoglycerol ether of *n*-octadecyl alcohol, was isolated from yellow bone marrow in 1941 by Holmes and co-workers, and a few reports have indicated that this compound possesses erythropoietic and leukopoietic activity. Similarities in chemical and physical characteristics of batyl alcohol and the thermostable plasma factor suggested a possible relationship between them.

The groups of normal rats were given 20 daily injections of either 12.5 mg. or 25.0 mg. of batyl alcohol in peanut oil, and a control group received peanut oil alone. The animals injected with the batyl alcohol developed reticulocytosis, erythrocytosis and myeloid erythrocytic hyperplasia. The newly formed erythrocytes were microcytic and showed decreased osmotic resistance. There was no increase in hemoglobin or hematocrit levels. This unique erythropoietic response was identical to that previously observed following the administration of the thermostable plasma erythropoietic factor. Batyl alcohol also induced thrombocytosis in the recipients. Although significant leukocytosis was not evident with the doses employed, other studies indicate that batyl alcohol has granulopoietic activity, but that a greater amount is required to evoke this response than is needed to induce erythrocytosis or thrombocytosis.

These observations are in accord with the hypothesis that all aspects of myelopoiesis are under the influence of humoral regulatory mechanisms. Hemoglobin synthesis appears to be governed by a relatively thermolabile factor. A thermostable, ether-soluble factor, which may be batyl alcohol or some related compound, accelerates erythroblastic cellular division and may also exert some degree of control over thrombopoiesis and granulopoiesis.

A Study of the Dynamics of Strontium and Calcium Metabolism and Radioelement Removal. W. B. LOONEY, C. J. MALETSKOS, MARIE HELMICK, JOHN REARDON, JONATHAN COHEN, and WARREN R. GUILD, Boston, Mass. (Introduced by Paul Zamecnik).

Ca^{40}, Ca^{45}, Sr^{85}, and Sr^{89} have been removed from the plasma of 18 dogs by circulating the blood for 1.5 to 7 hours through either an ion-exchange column or the artificial kidney. These procedures starting one hour after radioisotope administration removed 30 to 40 per cent of the injected dose; starting 0.5 to 3 days after administration removed 2.5 to 11.8 per cent of the injected dose; and starting 7 to 10 days after administration removed 0.24 to 2.4 per cent of the retailed dose. The mathematical analysis of the removal of the isotopes in nine dogs, starting one hour after administration demonstrated that 80 to 90 per cent of the dose was in a hypothetical compartment of bone which had a half-time of removal of 8 to 16 hours; the remainder was in a compartment approximating in size the extracellular space. The half-time removal from this compartment was 15 to 30 minutes.

The maximum sustained rate of mobilization of Ca^{40} from the skeletons of 10 dogs was in the order of 1 to 2 mg. per minute. During this time the serum calcium concentration was maintained constant at 1.5 mEq. per L.

The reduction in specific activity of tail bone segments taken at varying intervals during the removal period indicated that radiostrontium and radiocalcium were being removed from bone. Maintaining serum calcium concentrations by infusing Ca^{40} in the venous return did not alter the rate of radiostrontium removal, suggesting that strontium equilibrium is not primarily influenced by co-existing calcium levels. There was in general a reduction in renal excretion of both stable and radioactive cation during the period of radioelement removal. The reduction in the clearance of strontium was by as much as a factor of 20 to 30 while the reduction in the clearance of Ca^{40} and Ca^{45} was usually by a factor of 5. Reduction of the fecal excretion of radiostrontium and calcium was more marked than urinary excretion.

The application of this technique to the clinical removal of bone seeking radio elements will be discussed.

Isolation of the Rheumatoid Factor. JOSEPH LOSPALLUTO, ARTHUR LEWIS, and MORRIS ZIFF,* New York, N. Y.

A gamma globulin factor in the serum of most patients with rheumatoid arthritis has been under considerable study. This protein (rheumatoid factor) may be detected by a variety of methods, all of which involve reaction between the factor and other gamma globulins. Activity is manifested by agglutination of sensitized erythrocytes or latex particles or by formation of precipitates with Fraction II.

To isolate the factor, ion exchange chromatography (Sober and Peterson) was employed. Rheumatoid serum or euglobulin was applied to columns of diethylaminoethyl-cellulose (DEAE-cellulose), an anion exchanger, and fractionated by elution with buffers of decreasing pH and increasing concentration. Proteins fractionated in this manner appear in effluents in order of decreasing isoelectric point, gamma globulin being the first component to be eluted in a fraction recovered at pH 7. In the region of pH 5, however, we have observed the appearance of a second gamma globulin component distinct from the main bulk of gamma globulin. The rheumatoid factor was consistently recovered in this fraction. Since the rheumatoid factor is a macroglobulin, this observation indicated that the anion exchanger separated gamma globu-

lins on the basis of molecular size. Additional evidence for this has been obtained from ultracentrifugal experiments. This unusual chromatographic behavior has made possible the isolation of the rheumatoid factor in a highly purified state.

The factor is first precipitated from serum by the addition of normal Fraction II and the precipitate redissolved in 4 M urea. This solution is chromatographed on DEAE cellulose. Two components appear, one at pH 7 and a second at pH 5. The first is normal gamma globulin which can replace Fraction II as precipitant for the factor. The second is gamma globulin which is precipitated by Fraction II in a concentration of approximately 25 μg. per ml., agglutinates sensitized sheep cells in a concentration of approximately 1 μg. per ml., and demonstrates other reactions of the rheumatoid factor in high titer.

Penicillin Treatment of Experimental Infections Produced by Penicillin-Resistant Staphylococci. DONALD B. LOURIA, New York, N. Y. (Introduced by David E. Rogers).

Whether penicillin can affect the course of penicillin-resistant staphylococcal infections has been a subject of considerable controversy. In an attempt to gain information on this problem, experiments have been performed in which penicillin has been used to treat an experimental mouse infection produced by a strain of staphylococci insusceptible to high concentrations of penicillin *in vitro*.

Although eradication of the infection was not achieved, it could be demonstrated that penicillin treatment produced a significant increase in survival time even when given in dosages which resulted in blood levels well below the *in vitro* sensitivity of the microorganism. When larger concentrations of penicillin were administered, producing blood levels exceeding the *in vitro* sensitivity of the microorganism, the majority of animals were protected from death. Quantitative cultures performed on surviving mice revealed a marked decline or apparent eradication of kidney populations of staphylococci.

The protective effect was most marked when penicillin was given within six hours. When therapy was delayed for 24 hours, allowing early renal abscess formation to occur, protection was significantly reduced.

In vitro studies suggest a possible explanation for these results. When relatively small numbers of staphylococci were exposed to penicillin, significant killing was demonstrated at low penicillin concentrations. However, when the inoculum was increased to levels approximating those found in staphylococcal abscesses, no killing occurred despite massive penicillin concentrations.

These studies suggest that penicillin treatment may modify experimental infections produced by penicillin-resistant staphylococci when organ titers are relatively low. The insusceptibility of large staphylococcal populations to massive concentrations of penicillin suggests that staphylococci in abscess cavities may be relatively unaffected by penicillin therapy. The small protective effect of delayed penicillin treatment may thus be due to prevention of blood stream seeding rather than destruction of microorganisms within established abscess cavities.

Pressure-Volume Relationships of the Lung in Patients with Cardiorespiratory Diseases. FRANK W. LOVEJOY, Rochester, N. Y. (Introduced by Lawrence E. Young).

A method has been devised of recording both transpulmonary pressure and tidal volume on the X–Y axes of an oscilloscope. The resulting loop is photographed with a Polaroid camera. Compliance of the lung can be calculated from the point of zero velocity of the loop. The elastic work during inspiration can be estimated from the area of the right-angled triangle of which the compliance line is the hypotenuse. Nonelastic work is represented by the area of the loop. The total work of breathing is considered to be the area of the loop plus any part of the triangle outside the loop. Elastic and nonelastic work are related as a fraction of total work. The resulting work ratios have been compared with a battery of pulmonary function tests in 30 patients.

Values for compliance and the work ratios so obtained were similar to the values for these parameters obtained from loops constructed from pneumotachygraph records and esophageal pressures. The ratios of elastic work to total work and of nonelastic work to total work correlated very significantly with the one second timed vital capacity, maximum breathing capacity (per cent of predicted), ratio of residual volume to total capacity and with the air velocity index. There was no correlation of the work ratios to vital capacity or compliance. Compliance was usually reduced in patients with pulmonary emphysema or heart disease. In emphysema, the ratio of nonelastic work to total work was always markedly increased with a concomitant reduction of elastic work. In heart disease, the pressure-volume loops were relatively normal, but compliance was reduced.

This simple method of recording pressure-volume loops and the resulting work ratios is at least equal to a battery of pulmonary function tests in explaining the dyspnea of patients with cardiorespiratory disease.

Urinary Excretion of Indoles Causing False-Positive Watson-Schwartz Tests for Porphobilinogen. GEORGE D. LUDWIG, Philadelphia, Pa. (Introduced by Francis C. Wood).

Porphobilinogenuria, a pathognomonic sign of acute porphyria, is commonly detected by means of the Watson-Schwartz test, with formation of a chloroform-insoluble red complex with Ehrlich's benzaldehyde reagent (EBR). Chromogens responsible for occasional false-positive reactions have never been satisfactorily identified. These investigations were prompted by finding positive tests in conditions in which porphyria was simulated but excluded by *1*) quantitative porphyrin assays, *2*) evidence confirming other diseases, and *3*) proof by chemical, chromatographic, and spectrophotometric studies that the chromogen was not porphobilinogen (PBG) but an indolic compound.

In one such patient with hyperpigmentation, hepatic and neurological dysfunction, and dark urine, hemochromatosis was confirmed by iron studies, hepatic biopsy, and autopsy findings. The chromogen proved to be melanogen and melagin was isolated from the urine. Proof that melanogen (5,6-dihydroxyindole) can produce a positive Watson-Schwartz test was provided by synthesizing this compound from DOPA. In contrast to unsubstituted indole, which forms a chloroform-soluble pigment, 5,6-dihydroxyindole yielded an aqueous-soluble red aldehyde complex with a broad absorption maximum at about 556 mμ. The additional absorption peak at 520 mμ, characteristic of PBG-aldehyde complex, was absent. Subsequently, a positive Watson-Schwartz test was obtained with urine containing melanogen from a patient with metastatic melanoma.

Porphyria was excluded in another patient who manifested acute mental changes, hepatic dysfunction, cutaneous lesions, and a positive Watson-Schwartz test. A positive urorosein reaction and chemical evidence of increased indoleacetic acid and indican excretion was found. Two-dimensional paper chromatography confirmed these findings and demonstrated the presence of an additional unidentified indole. The patient's history and clinical features suggested pellagra, and dramatic response to nicotinamide was obtained with disappearance of excessive indoluria.

Urine from two patients with carcinoid syndrome gave negative tests, although higher concentrations of indoleacetic and 5-hydroxyindoleacetic acids yielded pink aqueous-soluble EBR complexes.

Excretion of Formiminoglutamic Acid in Folic Acid Deficiency States. A. LEONARD LUHBY, JACK M. COOPERMAN, DAVID N. TELLER, and ALVIN M. DONNENFELD, New York, N. Y. (Introduced by Milton Mendlowitz).

It has been demonstrated that in folic acid (PGA) deficiency, a major biochemical defect is the inability of the organism to degrade formiminoglutamic acid (FIGLU) to glutamic acid through the enzymatic transfer of the formimino (CH = NH) group. Broquist and Luhby have shown that in PGA deficiency induced by folic acid antagonist therapy in humans, FIGLU accumulates in the urine.

The following studies were undertaken to determine whether other PGA deficiency states could be recognized by the urinary excretion of formiminoglutamic acid.

Clinical subjects were patients with a variety of anemias suspected of being at least partially due to PGA deficiency. All initial urines showed no FIGLU excretion and each subject was given a metabolic load of 8 to 12 grams per day of the amino acid from which formiminoglutamic normally arises. Normal control subjects did not excrete FIGLU under these conditions. FIGLU was determined by microbiological assay using *Lactobacillus arabinosus.*

Results showed that in moderate macrocytic anemia of pregnancy, which did not respond to parenteral vitamin B_{12} and intravenous iron therapy, large amounts of FIGLU were excreted. PGA instituted while the metabolic loading was continued abolished the FIGLU excretion and corrected the anemia. In cases of megaloblastic anemia associated with sprue or malabsorption syndrome, FIGLU was also excreted. However, no FIGLU was found under these conditions in patients with Addisonian pernicious anemia in relapse, anemia associated with malignancy and iron deficiency anemia.

In several patients with anemia and toxemia of pregnancy, exhibiting no blood or marrow evidence of megaloblastosis, FIGLU was excreted. In one patient to whom two 10 milligram daily doses of folic acid were administered while the metabolic loading was continued, the FIGLU excretion was abolished, but reappeared when the PGA was discontinued.

The excretion of FIGLU in these patients demonstrates biochemical PGA deficiency. It can be used as a new clinical criterion of such states.

Observations on the Physiologic Changes Accompanying Hyperlipemia in Rabbits. WILLIAM S. LYNN, CARL WEBER, ROSE H. BROWN, MARGARET WELCH, and ASHTON B. MORRISON, Durham, N. C. (Introduced by Grace P. Kerby).

Experiments designed to study the physiologic effects of hyperlipemia and the mechanisms by which the hyperlipemia may be corrected were performed on rabbits fed diets containing 1 per cent amorphous cholesterol.

Rabbits with blood cholesterol values of 1,000 mg. per cent or higher were shown to drink and excrete two to three times as much water as animals fed stock diets. The rate of urinary (but not fecal) excretion of tracer doses of K^{42}, $S^{35}O_4$, P^{32} and Ca^{45} was two to four times greater than the controls. Na^{22}, however, was excreted at a slower rate. Urinary acid mucopolysaccharides precipitated by quaternary ammonium bromide and characterized by paper chromatography were similarly increased, as was the rate of incorporation thereinto of intraperitoneally injected $S^{35}O_4$. If the hyperlipemia persisted for three months or longer, excretion of all of the urinary components mentioned lessened. Pathologically the animals showed arcus senilis, aortic atherosclerosis and a very characteristic renal lesion consisting of a 2 to 3 mm. halo of extracellular fat at the corticomedullary junction, extending into the medulla.

Factors which facilitated clearing of the high blood fat after cessation of sterol ingestion were increased water intake (600 to 1,000 ml. daily) or addition of KCl (0.5 per cent) and K_2HPO_4 (0.5 per cent) to the drinking water. Factors which retarded the clearing process included dietary addition to saturated or unsaturated fats (from 1 to 20 per cent of the diet) or addition of glucose (7 per cent) to the drinking water.

Observations on the ability of these hyperlipemic animals to oxidize trace amounts of tube-fed saturated and unsaturated radioactive fatty acids to CO_2 indicated two facts: *1*) Unsaturated fatty acids (linoleic and linolenic) were oxidized more rapidly than stearic, and *2*) the hyperlipemic animals oxidized all three acids at a re-

duced rate. However, mitochondria (kidney or liver) from these animals oxidized fatty acids to CO_2 normally.

Thus, dietary hyperlipemia in the rabbit has been shown to produce profound effects on water, salt, polysaccharide and fat metabolism.

The Intracellular Osmolarity of Mammalian Tissues. LeRoy H. Maffly and Alexander Leaf,* Boston, Mass.

Because of its clinical importance in determining fluid distribution in patients as well as its basic importance to the regulation of cell volume, there has been considerable interest in learning the effective osmolarity within cells. Previous investigators have reported that both the freezing point and melting point depression of various mammalian tissues is one and one-half to two times greater than that of serum. From this it has been concluded that the osmolarity of the intracellular fluid is considerably greater than that of extracellular fluids. The methods used, however, have been criticized because of autolysis which will occur during the interval between removal of the tissue and the endpoint of the measurement.

In the present work, melting point measurements were made on tissue frozen in liquid nitrogen immediately upon removal from anesthetized animals. The frozen tissue was then pulverized and suspended in chilled, inert, liquid silicone. The suspension was then rapidly stirred and its temperature increase measured by a thermistor.

The theoretical melting curve of a solution was shown to be a rectangular hyperbola. Actual tissue melting curves were found to be closely superimposable on the theoretical curve. When the melting curves of various tissues (muscle, liver, heart and brain) were compared with the animals' serum, no significant differences were found. Whole kidney was approximately 10 per cent more concentrated than serum, a difference attributed to its content of hypertonic urine. When frozen tissue was exposed to room temperature for 10 or 20 minutes the subsequent measurement showed a marked increase in osmolarity. This confirms that rapid autolysis will lead to spurious results.

These direct measurements demonstrate that the osmolarity, or more correctly the water activity, within mammalian cells is the same as that of the extracellular fluids.

Adenosine Triphosphate Generation in Relation to the Pathway of Glucose Utilization in Human Erythrocytes. Paul A. Marks, Anne B. Johnson, and Julia Banks, New York, N. Y. (Introduced by Alfred Gellhorn).

Adenosine triphosphate (ATP) formation was studied in relation to the pathways of glucose utilization in mature human erythrocytes (RBC). The Embden Meyerhof (EMP) and pentose phosphate pathways (PPP), but not the Krebs cycle, are active in RBC. ATP generation may be coupled, in the EMP, to oxidation of 3-phosphoglyceraldehyde or electron transfer from reduced diphosphopyridine nucleotide (DPNH) and, in the PPP, to electron transfer from reduced triphosphopyridine nucleotide (TPNH). ATP concentrations were determined under conditions of increased and decreased glucose breakdown via EMP or PPP.

Stimulation of RBC glycolysis by addition of glucose resulted in markedly higher levels of ATP. Blocking glycolysis via the EMP with iodoacetic acid (IAA) caused rapid decreases in ATP concentrations to undetectable levels (less than 10^{-6} M per Gm. hemoglobin). IAA neither inhibited glucose-6-p dehydrogenase activity nor glucose oxidation via the PPP.

A relationship between ATP levels and glycolysis via the EMP, but not via the PPP, was further indicated by the following findings: *1)* RBC with an hereditary deficiency in glucose-6-p dehydrogenase had markedly decreased rates of oxygen (O_2) consumption and somewhat reduced rates of 1-C^{14}-glucose oxidation to $C^{14}O_2$ but normal ATP levels. *2)* RBC stored in heparinized plasma for two days had little or no detectable ATP although their ability to oxidize 1-C^{14}-glucose to $C^{14}O_2$, a reaction associated with TPNH generation, decreased only 30 to 40 per cent.

In addition, methylene blue concentrations resulting in a ten- to twentyfold rise in RBC O_2 consumption increased 1-C-^{14}-glucose oxidation to $C^{14}O_2$ but had no effect on ATP concentrations.

These data indicate that ATP generation, essential to the maintenance of RBC integrity, is dependent on glucose utilization via the EMP. The fact that ATP generation in RBC is independent of O_2 consumption suggests that its formation is linked primarily to the oxidation of 3-phosphoglyceraldehyde and not of DPNH or TPNH.

Relationship Between Red Cell and Plasma Volume During Recovery from Pernicious Anemia and Megaloblastic Anemia of Folic Acid Deficiency. Ralph W. Massie, Nashville, Tenn. (Introduced by Hugh J. Morgan).

There is poor correlation between the prompt reticulocytosis which appears after initial treatment of Addisonian pernicious anemia and the frequently delayed elevation of hematocrit. This could be explained by concomitant increases in red cell volume and in plasma volume during the early phase of recovery. Thus, equiproportional increases in red cell volume and plasma volume would not alter the hematocrit. Since 1900, many techniques have been employed to measure the blood volume in pernicious anemia and very conflicting values have been reported Low, normal and rarely even elevated values have been observed in untreated patients. Frequent determinations of blood volume during convalescence have apparently never been performed. The reported data, however, are conflicting.

Three patients with pernicious anemia and one patient with megaloblastic anemia secondary to folic acid deficiency were studied. Frequent blood volume determinations were performed by dilution of chromium^{51} labeled

erythrocytes. Reticulocyte counts and hematocrits were performed daily.

The blood volume in severe untreated pernicious anemia and megaloblastic anemia of folic acid deficiency is at times markedly diminished. Part of the deficit of blood volume is a deficit in plasma volume. The routine hematocrit determinations in this situation fail to reveal the profound deficits in circulating red blood cells that exist.

The increase in red blood cell volume during the first three weeks of treatment is rapid and almost linear.

During the first two weeks of treatment, the blood volumes returned to normal or slightly above normal. Both plasma and red cell volumes increased during this time.

The failure of the hematocrit to rise in proportion to the increase in total red cells is due to an early equiproportional rise in plasma volume.

The final rise in hematocrit is accompanied by a decrease in plasma volume, with the total blood volume remaining constant during this stage of recovery.

The Nature of the Sodium Diuresis Produced by an "Aldosterone Antagonist," Spirolactone. WALLACE W. MCCRORY and WALTER R. EBERLEIN, Philadelphia, Pa. (Introduced by Henry L. Barnett).

A new synthetic steroid (SC-8109, Searle; spirolactone), described as an aldosterone antagonist, was administered orally in equivalent dosage (25 mg. per Kg. body weight) to four children free of renal or adrenal disease, and to a 12 year old male with chronic hypokalemic alkalosis who had been shown not to have hyperaldosteronism by means of urinary aldosterone assays and exploratory laparotomy. Renal function of the latter subject was normal as judged by renal clearance measurements (C_{in}, C_{pah} and Tm_{pah}), urinary solute concentrating ability and response to a five day load of ammonium chloride (175 mEq. per 1.73 $M.^2$ surface area per day). Urine was collected from each subject for 48 hours before and 48 to 72 hours after administration of the synthetic steroid. The rate of excretion of sodium, potassium, chloride, titratable acid and ammonia and the urinary pH were measured. All subjects were maintained on a normal salt intake. The administration of the synthetic steroid resulted in an increased urinary sodium excretion, an increase in urinary pH and a decrease in excretion of titratable acid in all subjects. The urinary excretion of potassium remained unchanged in the control subjects. The patient with alkalosis, however, excreted significantly less potassium and chloride. These results are indicative of altered renal tubular hydrogen ion excretion and suggest that the steroid induces sodium excretion by mechanisms other than aldosterone antagonism *per se*. The effects of prolonged administration of spirolactone to normal subjects and the patient with unexplained alkalosis will be described.

A Mechanism of Renal Hypertension in the Human. JOHN P. MERRILL,* ALBERTO GUINAND-BALDO, and CARMELO GIORDANO, Boston, Mass.

Recent experimental work in animals has suggested that renal hypertension may be associated with the absence of some metabolic or excretory function of the kidney rather than its production of a pressor substance. A unique opportunity to test his hypothesis in the human has been provided by observations on identical twins with hypertension and renal disease in whom a third normal kidney has been successfully transplanted. Four patients with severe renal failure and hypertension received a successful renal homograft from an identical twin. In all patients the blood pressure decreased rapidly toward normal at a time when the patient possessed two severely damaged but one normal kidney. The average blood pressure preoperatively was 210/140; two weeks postoperatively, 150/95. Surgical stress of a similar magnitude (*i.e.*, the transplantation of a cadaver kidney as a true homograft in another patient with hypertension) did not result in decrease in blood pressure. The rate of blood pressure decrease in the twins was not dependent upon changes in weight or sodium balance but roughly correlated with the rapidity with which the transplanted kidney function improved. In two patients the infusion of *l*-norepinephrine at constant rates before and after the removal of the two damaged kidneys was studied. The healthy identical twin donor served as control. Before transplantation the response to norepinephrine was markedly greater than in the normal twin. The addition of a third but normal kidney markedly decreased the blood pressure "ceiling" to norepinephrine, although it remained higher than in the control twin. Both before and after transplantation this effect was enhanced by a low sodium diet. These findings suggest that the kidneys may play a role in hypertension by modifying the response to normally occurring pressor amines.

Increased Binding of Vitamin B_{12} by Serum Mucoproteins in Chronic Myelogenous Leukemia. AARON MILLER and JOHN F. SULLIVAN, Boston, Mass. (Introduced by C. P. Emerson).

The serum in chronic myelogenous leukemia (CML) has an increased binding capacity for vitamin B_{12} both *in vivo* and *in vitro*. We have studied the *in vitro* binding of added cobalt^{60}-labeled vitamin B_{12}^* by normal and CML sera with dialysis techniques.

B_{12}^* binding occurred within one minute and thereafter did not exchange with added carrier B_{12}. Binding was unaffected by pH changes from 6 to 12 but was decreased at lower pH levels (pH 3.2, normal sera binding decreased 77 per cent, CML sera 10 per cent). A large fraction (average, 69 per cent) of added radiovitamin "bound" by normal sera after dialysis at pH 7.3 was measured as "unbound" by *Euglena gracilis* assay whereas a smaller fraction (average, 11 per cent) was so measured in CML sera. At the pH of this assay (3.5) 48 per cent of the radiovitamin which had been bound in normal sera at pH 7.3 became dialyzable compared to 10 per cent in CML sera. Prior addition of pseudovitamin B_{12} or hydroxo-cobalamin decreased B_{12}^* binding in both

sera; dimethylbenzimidazole, cyanide or agents combining with sulfhydryl groups had no effect.

Normal serum mucoproteins, remaining after sulfosalicylic acid precipitation of proteins, bound an average of 0.24 millimicrograms B_{12}^* per milliliter (13 per cent of total serum binding). Binding by CML mucoproteins was increased (5.14 millimicrograms B_{12}^* per milliliter or 59 per cent of total serum binding), which entirely accounted for increased B_{12}^* binding in some CML sera. CML mucoprotein B_{12}^* binding was unchanged at pH 3.2. Precipitation of CML mucoprotein-B_{12}^* complex with phosphotungstic acid resulted in 95 per cent recovery of radiovitamin in the precipitate.

The observed differences in vitamin B_{12} binding between normal and leukemic sera in respect to pH change and *Euglena gracilis* assay may be explained by mucoprotein binding of vitamin B_{12}. The increased serum vitamin B_{12} binding in chronic myelogenous leukemia may be entirely due to this mucoprotein binding.

Hemolysis in Uremia: Prevention of Intracorpuscular Defect by Renal Tissue. E. E. MUIRHEAD * and J. A. STIRMAN, Dallas, Texas.

Under certain conditions a hemolytic component may contribute greatly to the anemia associated with renal disease and uremia. Hemolysis has been studied in uremia.

Erythrocytes obtained four days following bilateral nephrectomy of the dog were transfused into recipients compatible by the antiglobulin technique and the life span was measured simultaneously by the Ashby and radiochromium methods. Comparable results indicating a shortened life span were obtained by the two methods. This approach supported the use of the radiochromium method under these conditions.

Erythrocytes obtained four days following bilateral nephrectomy of dogs were transfused into normal recipients on 30 occasions. A prominently shortened life span was determined. The results differed significantly from those obtained when the erythrocytes of normal dogs were reintroduced into their normal environment or when these cells were transfused into normal recipients. In seven experiments the same shortened life span was observed in the nephrectomized dog as in the normal recipient. These findings indicated an intracorpuscular defect. By this method this defect reached a maximum 24 hours following the nephrectomy.

In five experiments the ureter was connected to the vena cava and the opposite kidney was removed. Four days later the erythrocytes from these animals were transfused into normal recipients. The life span approached normal values. There was a significant difference between the life span curves following nephrectomy and those following ureterocaval anastomosis.

The results indicated the rapid development of an intracorpuscular defect following nephrectomy. Under the same degree of excretory renal insufficiency (uremia) protection against such a defect by intact renal tissue was demonstrated. Thus the stability of the erythrocytes in the circulation of the dog appears to be due in part to nonexcretory renal function(s).

The Effect of Potassium Depletion and Repletion on Aldosterone Excretion. ALEX F. MULLER, ELIZABETH L. MANNING, and ANNE M. RIONDEL, Geneva, Switzerland. (Introduced by Marian W. Ropes).

The mechanism by which potassium loading increases and potassium depletion decreases urinary aldosterone has not been established. Some claim that changes in serum potassium regulate hormone activity since serum sodium and the state of hydration could not be correlated with the observed variations in aldosterone. The observation that urinary aldosterone increases with potassium loading without changes in external sodium balance and serum potassium has led some workers to infer that the potassium ions act directly on hormone production. More recent experiments established a close relationship between the effects of potassium and body fluid shifts; yet the authors concluded that their studies were not consistent with the hypothesis that all changes occurring in aldosterone secretion during potassium depletion and repletion were regulated by extracellular fluid volume.

The present metabolic studies were carried out in normal subjects in an attempt to elucidate the various factors involved in the regulation of aldosterone during potassium depletion and repletion. Hypokalemia was produced either by administering exchange resins or by withdrawing potassium from the diet.

The diminished aldosterone output was always accompanied by relative sodium retention and hemodilution as measured by hematocrit and total protein changes. Further expansion of the plasma and extracellular fluid will occur with increasing sodium intake despite a falling urinary aldosterone. When potassium loading produces hemoconcentration by a shift or a loss of sodium and water, only then does aldosterone rise. Sodium excretion may increase despite rising urinary levels of aldosterone.

The changes in urinary aldosterone occurring during these experiments can be explained on the basis of variations in the intravascular volume without invoking a direct effect by the potassium ion.

The Relation of Thyroidal Hormone Level to Epinephrine Response. JOHN F. MURRAY and JOHN J. KELLY, JR., Brooklyn, N. Y. (Introduced by William Dock).

The hemodynamic and metabolic responses to epinephrine are strikingly altered by variations in the level of the thyroid hormones. Little clinical use has been made of the phenomenon of increased sensitivity of the hyperthyroid patient to epinephrine because no previous study attempted a quantitative separation of the hyperthyroid from the euthyroid response to epinephrine.

Intravenous epinephrine was administered to 28 euthyroid subjects, 22 normal and 6 hypermetabolic, and to 11 patients with thyrotoxicosis. Differences in the magnitude of increase of the oxygen consumption, pulse rate, and blood pressure clearly separate these groups. Nor-

mals tolerated infusion rates of epinephrine up to 0.2 micrograms per Kg. per minute before experiencing symptoms of epinephrine toxicity. Hyperthyroid patients could only withstand a fraction of this dose. The sensitivity of the thyrotoxic subject to epinephrine is not due to hypermetabolism because the response of the hypermetabolic but euthyroid group was no greater than that of the normals.

Analysis of the normals' and thyrotoxic subjects' response to an infusion rate of epinephrine of 0.05 μg. per Kg. per minute was made. This dosage was chosen because it was capable of producing striking changes in the hyperthyroid subjects and yet not likely to produce disturbing symptoms. The mean change of O_2 consumption in the normal group was +5 per cent with an S.D. of $\pm$ 5 per cent. Three of 14 values of the hyperthyroid group fell within one standard deviation of this.

The increase in heart rate times pulse pressure provided a sharper separation of the two groups. The mean for the euthyroid was +25 per cent with an S.D. of $\pm$ 12 per cent with no values from the hyperthyroid group falling within two standard deviations.

The epinephrine test is a simple test which appears to provide a high degree of specificity in distinguishing hyperthyroidism from other conditions.

Studies on the Influence of Cortisone on Serum Calcium Homeostasis. W. P. LAIRD MYERS and WALTER LAWRENCE, JR., New York, N. Y. (Introduced by David A. Karnofsky).

Balance studies have demonstrated that cortisone may induce a negative calcium balance but these studies have not aided in understanding the role of the adrenals in homeostatic mechanisms governing the serum calcium level. Control of this level has been defined in terms of an equilibrium existing between bone and extracellular calcium. That the adrenals may exert an influence on this equilibrium is suggested by the occasional occurrence of hypercalcemia in adrenal insufficiency in both man and experimental animals.

The present investigations, therefore, were undertaken to define more precisely the role of cortisone in serum calcium homeostasis: *1*) Cortisone administration to a patient with hypoparathyroidism (controlled by vitamin D and calcium lactate) caused hypocalcemia with no change in the calcium balance. *2*) The influence of cortisone withdrawal was studied in an adrenalectomized, thyro-parathyroidectomized dog. Coincident with the development of adrenal insufficiency, hypercalcemia was noted. These changes were reversed when cortisone was again administered. This study was repeated under balance conditions with the same results but with no appreciable alteration in the consistently negative calcium and phosphorus balances. *3*) Cortisone has been reported as having produced hypercalcemia in nephrectomized dogs. The effect of cortisone was therefore studied in a nephrectomized dog who had undergone prior thyro-parathyroidectomy and under these circumstances hypercalcemia did not occur.

These observations suggest that cortisone, in addition to its action on calcium balance, may also exert an influence on the equilibrium between bone and extracellular calcium in a direction opposite to that of parathormone and vitamin D. The fact that hypocalcemia does not develop ordinarily with cortisone administration may be accounted for by compensatory activity of the parathyroids. That cortisone lack can lead to hypercalcemia in the dog in the absence of the parathyroids indicates that its action is not that of direct antagonism to parathormone.

The Demonstration of a Succinoxidase Inhibitor in Gastrointestinal Mucosa. M. NAKAMURA, P. PICHETTE, S. BROITMAN, A. BEZMAN, N. ZAMCHECK, and J. VITALE, Boston, Mass. (Introduced by Charles S. Davidson).

The study of the gut mucosa was stimulated by the hitherto unexplained variability of the succinoxidase activity of the rat intestinal mucosa despite its relative constancy in other tissues such as heart, liver, and kidney. Thus, the oxidation of succinate [$QO_2(N)$] by intestinal mucosa of 41 rats varied from zero (30 rats) to 50 to 100 (7 rats) to 100 to 210 (4 rats). On the other hand, xanthine oxidase activity of rat intestinal mucosa was quite constant, and succinoxidase of guinea pig intestinal mucosa was similarly constant.

The addition of rat intestinal mucosal homogenate caused a decrease in oxygen uptake of guinea pig intestinal mucosa. These observations suggested that an inhibitor of the succinoxidase system resided in the intestinal mucosa of rats and was absent from that of guinea pig. Similarly, the oxygen uptake of rat heart homogenate in the presence of succinate was decreased by the addition of rat intestinal homogenate. On the other hand, guinea pig homogenate did not affect the activity of rat heart homogenate. In general, the lower the enzyme activity of a given rat intestinal mucosa, the greater its inhibition of heart enzyme activity. The addition of rat serum to rat intestinal homogenate increased oxygen uptake. Similarly, serum reversed the inhibitory effect of the intestine on heart enzyme.

The inhibitor, soluble in alkali and fat solvents, proved to be a fatty acid. Fatty acids are normally found in the intestinal mucosa of the rat. Rat heart succinoxidase activity was also inhibited by rat gastric mucosa. Human gastric mucosa has failed to date to demonstrate any inhibitory effect on rat heart succinoxidase activity.

Failure of Aldosterone to Maintain Sodium Retention in Normal Subjects and Addisonian Patients. DON H. NELSON * and J. THOMAS AUGUST, Boston, Mass.

Initial sodium retention followed by sodium diuresis has been reported in men and dogs receiving desoxycorticosterone or fluorohydrocortisone. This effect has not been reported in patients with Addison's disease or in normal subjects receiving aldosterone. Sodium diuresis occurring with extracellular fluid volume expansion has been attributed to a decrease in endogenous aldosterone produc-

tion or possibly to increased secretion of other adrenal steroids. Two normal subjects were given 3 to 6 mg. of *dl*-aldosterone diacetate in sesame oil intramuscularly daily in divided doses for 14 to 26 days while being maintained on a constant diet. Initial marked sodium retention with weight gain of 2 to 3 Kg. was followed by a spontaneous increase in sodium excretion and no further gain in weight during the remaining period of aldosterone administration. All subjects showed sodium retention in excess of water retention without an increase in plasma sodium concentration. There was no significant increase in urinary 17-hydroxycorticoid excretion during the period of increased sodium excretion but secretion of adrenal steroids not measured by the techniques employed could not be ruled out. Aldosterone was then given to two well-documented Addisonian patients on constant cortisone therapy, one of whom in addition received vasopressin (Pitressin®). Both patients showed a response similar to the normal subjects', and a natriuresis of over 300 mEq. occurred in the Pitressin® treated subject without diminution of urinary output or further weight gain. These data indicate that in normal subjects and cortisone treated Addisonian patients, sodium diuresis may occur during volume loading despite continued administration of aldosterone.

The Effect of Parathyroidectomy on the Availability of Bone Sodium. GEORGE NICHOLS, JR. and NANCY NICHOLS, Boston, Mass. (Introduced by K. Emerson, Jr.).

Previous work by the authors and others indicates that sodium moves out of bone mineral into the extracellular fluid in response to acute sodium depletion and/or metabolic acidosis. We have postulated that this might result in a substitution of calcium for sodium at the surface of bone crystal as suggested by Neuman's *in vitro* studies of hydroxyl apatite. Citrate appears to be concerned in mobilizing calcium from bone into extracellular fluid, and the production of this acid by bone is enhanced by parathyroid hormone. These findings suggested that parathyroidectomized animals would have normal or low bone sodium and that acute sodium depletion might increase their susceptibility to tetany due to migration of calcium from extracellular fluid into sites in bone crystal previously occupied by sodium.

To test this hypothesis, plasma and bone from normal and parathyroidectomized rats were analyzed for water, sodium, chloride, calcium, and plasma pH. Animals from each group were sodium depleted by intraperitoneal dialysis against ammonium chloride solution 24 hours prior to sacrifice. Parathyroidectomy produced a 47 per cent fall in plasma Ca but no change in sodium or pH. In contrast, bone sodium increased 8 per cent ($p < 0.001$) without any significant change in calcium. Sodium depletion of normal animals, confirming previous findings, caused plasma pH to decrease 0.05 without change in sodium or calcium, while bone sodium decreased 4 per cent. In parathyroidectomized animals pH decreased 0.10, plasma sodium fell significantly, and Ca rose 15 per cent. Furthermore, bone sodium remained unchanged and no tetany was seen.

These findings suggest that parathormone is necessary for the mobilization of sodium from bone mineral perhaps through unknown modifications in bone matrix or by the production of local acidosis due to increased citrate formation. Moreover, in bone, its action on sodium metabolism appears more important than its effect on calcium.

Experimental Inapparent Poliomyelitis: Factors Involved in Reinfection of Humans with Attenuated Poliovirus. JAMES C. NIEDERMAN, New Haven, Conn. (Introduced by John R. Paul).

Investigation of the use of attenuated strains of polioviruses has not only pointed the way to new methods of immunization against this disease but has also added considerable knowledge to the picture of subclinical inapparent poliomyelitis infection. The studies to be reported have been concerned with the capacity for reinfection in individuals repeatedly fed attenuated virus, and with the relation of such repeated infection to the maintenance of immunity. Major questions to be answered were: *a*) What is the role of the dosage of virus in reinfection, and *b*) what are the effects of pre-existing antibody levels in the host when rechallenged with poliovirus.

Eleven individuals, aged 6 to 16 years, who had previously been fed Sabin's attenuated LSc strain of Type I poliovirus, were rechallenged with the same large dose of the same virus 16 weeks after their first exposure. On this rechallenge, virus excretion in the stool occurred intermittently in all 11 individuals, but such excretion was shorter and less continuous than on initial feeding. A delayed antibody rise was induced in 5 individuals, all of whom had a prefeeding neutralizing antibody titer of 256 or less. Six individuals (most of whom had antibody titers of 256 or more) failed to develop antibody rises in spite of virus excretion.

In order to see how readily this reinfection could be repeated, these same 11 individuals and 5 others were again challenged with four different doses of the same attenuated virus, ranging from large to small. The results indicate that there is a direct relationship between the amount of virus fed and the ease of reinfection as measured by virus excretion. With large virus doses reinfection readily occurred, while with smaller doses reinfection infrequently occurred even though the antibody levels in the hosts were all approximately of the same order.

Blood Coagulation and Blood Lipids. SAUL NITZBERG, ROBERT GOLDSTEIN, and MICHAEL A. PEYMAN, Boston, Mass. (Introduced by Samuel Proger).

In fasting and postprandial specimens, we have studied whole blood clotting time in glass and silicone, clotting time or recalcified plasma, heparin tolerance test, thromboplastin dilution test, thrombo-elastography and Stypven time. The serum lipids measured were total fats, fatty acids, cholesterol, phospholipids, beta-lipoproteins, and occasionally ethanolamine phosphatide.

Generally, we have not confirmed reports of blood hypercoagulability following ingestion of fats. The exception was the Stypven time, which was usually decreased postprandially. The artificiality of this test, however, makes its value questionable. Also, there appeared to be a trend toward increased coagulability in some normal subjects postprandially, as shown by the heparin tolerance test and thrombo-elastography. However, this tendency was not constant on repeated testing.

There was no close correlation between changes in blood coagulability and the degree and character of the postprandial hyperlipemia as chemically determined.

Blood coagulability was not significantly different in 38 patients whose serum cholesterols were below 200 mg. per cent, as compared with 32 patients whose values were above 300 mg. per cent.

In 10 patients with hypercholesteremia, coagulation studies are being performed both when the serum lipids are elevated and when they are lowered after the daily ingestion of unsaturated fatty acids. The results will be reported.

Measurement of Coronary Blood Flow and Myocardial Rubidium Uptake With Rb^{86}. DAVID NOLTING, ROBERT MACK, ERNST LUTHY, MORTON KIRSCH, and CHARLES HOGANCAMP, St. Louis, Mo. (Introduced by R. J. Bing).

The dynamics of myocardial Rb^{86} uptake and its use in the estimation of coronary blood flow in dogs were studied. Myocardial extraction and uptake of Rb^{86} were determined with coronary sinus catheterization. Coronary blood flow, determined with nitrous oxide, served as reference. Myocardial extraction of Rb^{86} remained constant at changing arterial concentrations; as coronary blood flow decreased, the per cent myocardial extraction of rubidium rose. This suggests diffusion of Rb^{86} into a large miscible pool. Coronary blood flow using Rb^{86} was determined by the Fick principle or by the method of Love and Burch. Coronary A–V rubidium differences can be calculated without coronary sinus catheterization since the percentage extraction of Rb^{86} by the heart remains constant (approximately 43 per cent). Myocardial uptake of Rb^{86} can be calculated: a) from the slope of precordial radioactivity, since slope is proportional to the myocardial rubidium uptake [uptake equals flow (N_2O) times myocardial rubidium extraction]; and b) by a proportionality constant obtained from the ratio of precordial activity to radioactivity of heart homogenates obtained after termination of the experiment, or from precordial activity obtained after insertion of balloons filled with known concentrations of Rb^{86} into the ventricles. A straight line relationship exists between precordial count and specific activity of Rb^{86}. Values for coronary flow determined with nitrous oxide show good agreement with those derived from the use of Rb^{86} using the Fick principle; this is particularly the case at low coronary blood flows. The method based on the Fick principle appears to be of promise for the determination of coronary blood flow in man.

Studies of the Activities of Steroid Hormones on Electrolyte Balance and the Constituents of Protoplasm: The Effects of 16 Alpha-Hydroxylation of 21-Carbon Steroids. MAURICE M. PECHET,* EVELYN L. CARROLL, MARY MITCHELL, and MARY JO WEGNER, Boston, Mass.

Previous studies have demonstrated that 1,2-dehydrogenation of parent steroids, cortisone and hydrocortisone, results in an increase in anti-anabolic and anti-inflammatory activities and causes a decrease in sodium-retaining properties. 9α-Fluoronation of parent steroids causes a marked increase in sodium-retaining properties. The effects of 16α-hydroxylation on the metabolic activities and on the renal electrolyte activities of 1,2-dehydro-9α-fluorohydrocortisone were investigated with complete balance studies in Addisonian subjects.

1,2-Dehydro-9α-fluoro-16α-hydroxyhydrocortisone (9α-fluoro-16α-hydroxyprednisolone; triamcinolone) in doses of 30 mg. daily caused marked losses of nitrogen and phosphorus. The loss of phosphorus was commensurate with the loss of nitrogen. In doses of 20 and 30 mg. daily, there was 1) a prompt increase in excretion of calcium, 2) a loss of sodium, and 3) a marked loss of potassium. The loss of potassium was much greater than that predicted from the loss of nitrogen and was not accompanied by parallel changes in serum potassium.

In doses of 30 mg. daily 1,2-dehydro-9α-fluoro-16α-hydroxyhydrocortisone did not offset the sodium retention induced by 150 micrograms daily of 1,2-dehydro-9α-fluorohydrocortisone (dose ratio, 200:1); in doses of 20 mg. daily 1,2-dehydro-9α-fluoro-16α-hydroxyhydrocortisone did offset, after 48 hours, the sodium retention induced by 500 micrograms daily of aldosterone (dose ratio, 40:1).

Thus, 16α-hydroxylation of 1,2-dehydro-9α-fluorohydrocortisone results in a loss of the sodium-retaining property and a retention of the potassium-losing property of the parent steroid. Pronounced weakness and asthenia were associated with the administration of 1,2-dehydro-9α-fluoro-16α-hydroxyhydrocortisone (triamcinolone) and are probably due to potassium loss. Potassium loss persisting concomitantly with sodium loss has not been observed heretofore with steroids possessing anti-inflammatory properties.

Clinical Trials of Five Strains of Orally Administered Attenuated Polio Virus. STANLEY A. PLOTKIN, HILARY KOPROWSKI, KLAUS HUMMELER, AGNES FLACK, and JOSEPH STOKES, JR., Philadelphia, Pa. (Introduced by Frederick C. Robbins).

Five strains of attenuated polio virus have been used in the vaccination of a group of infants. The five strains used were: CHAT, (Type I), Calf (Type I), Jackson (Type II), P-712 (Type II), and Fox (Type III). All strains had been attenuated by passage through mouse brain, tissue culture, and chick embryo, and were the progeny of a single plaque. The vaccines were prepared in monkey kidney tissue culture and dispensed in tissue culture fluid diluted in milk. In none of the infants were

there any illnesses attributable to the viruses administered. Vaccinations with CHAT, Calf, P-712, and Fox strains were effective at widely varying dosages. Jackson strain, however, appeared to be a poor antigen.

The presence of high titers of passive maternal antibodies and/or young age seemed to exert an inhibiting effect on the process of vaccination. Age was not always important, as illustrated by six infants less than two weeks of age who were successfully vaccinated. Follow-up antibody determination on infants immunized with attenuated strains of living polio virus in 1955 and 1956 revealed persistence of antibodies for periods up to 28 months. Six infants were vaccinated against all three types of polio. When the strains were fed at three week intervals, no interference between strains was observed.

In a small town near Philadelphia, 18 families are participating in an open field trial of CHAT, P-712, and Fox strains. One child without antibodies was vaccinated in each family, while stools were collected from all members of the family in an effort to observe spread of fecal virus.

On the Electronmicroscopic Recognition and Clinical Cure of Lipoid Nephrosis in Adults. VICTOR E. POLLAK, GIUSEPPE FOLLI, CONRAD L. PIRANI, ROSS T. W. REID, and ROBERT C. MUEHRCKE, Chicago, Ill. (Introduced by Robert M. Kark).

Lipoid nephrosis is a nonfatal illness characterized by the nephrotic syndrome in which tubular but not glomerular changes would be found post mortem were the patient to die of a complicating disease. Of 96 adults previously studied by renal biopsy and light microscopy, 34 had glomerulonephritis (membranous or proliferative) and 11 had tubular disease with surprisingly little or no glomerular damage.

To elucidate this further, electronmicroscopic, clinical and metabolic observations were made on 10 nephrotics: three children with lipoid nephrosis, three adults with membranous glomerulonephritis (Ellis Type II, Longcope Type B), and four adults with little or no glomerular damage by light microscopy. Four healthy control subjects were studied. To insure adequate sampling five to seven glomeruli were studied for each biopsy. Serial studies were made after dextran or prednisone diuresis.

In membranous glomerulonephritis the lamina densa (basement membrane) was greatly thickened (eight times) and split longitudinally. Endothelial and epithelial cells were normal. Studies on children partly confirmed Farquhar's observations, *viz.*, the basement membrane and endothelial cells were normal, but the foot processes of the epithelial cells were fused into a continuous layer of cytoplasm, and many trabeculae were collapsed. An identical picture was observed in adults. After dextran diuresis in one patient epithelial cell abnormalities were unchanged. Four patients, treated with prednisone, diuresed. Epithelial cell healing was seen in second biopsies, which were completely normal when proteinuria had disappeared. Prednisone has been used for three years in one patient, who is well clinically, biochemically and electronmicroscopically, as are others treated for shorter periods.

These observations indicate that: *1*) Membranous glomerulonephritis and lipoid nephrosis are distinct disease entities. *2*) Lipoid nephrosis occurs in adults as well as children. *3*) Lipoid nephrosis is a reversible disease, at least in early stages. *4*) In lipoid nephrosis diuresis *per se* is not responsible for restoration of glomerular lesions to normal.

Determination of the Rate of Insulin Destruction In Vivo. THADDEUS E. PROUT and IUEAN E. EVANS, Baltimore, Md. (Introduced by Samuel P. Asper, Jr.).

Insulin disappears rapidly from the intravascular space following the intravenous administration of insulin labeled with iodine^{131} (I^{131}). The half-life of insulin I^{131} *in vivo* has been shown to be approximately 25 minutes. This rapid decline in the plasma concentration of insulin I^{131} has been attributed to the removal of insulin from the circulation by the binding of insulin to tissue and by proteolytic destruction of insulin, principally in the liver. Whether or not degradation accompanies tissue binding *in vivo* has not been clearly shown.

Twenty-two normal male rabbits have been given insulin I^{131} by intravenous injection. The rate of disappearance of insulin I^{131} has been followed by measuring the decline of radioactivity of the insulin separated from plasma by electrophoresis. The metabolism of the injected radioinsulin has been determined quantitatively by calculating the amount of free I^{131} which has been either separated from the injected insulin or excreted in the urine. The influence of crystalline-zinc insulin (CZI) in doses of 10, 100, and 1,000 units per Kg. on the rate of disappearance and metabolism of insulin I^{131} has been studied.

Approximately 90 per cent of the radioinsulin is metabolized during the 80 minutes following injection. Neither the quantity metabolized nor the rate of disappearance of radioinsulin from the serum is altered by the intravenous administration of CZI in doses of 10 or 100 units per Kg. The half-life of the radioinsulin is increased to 53 minutes and metabolism is decreased to 48 per cent in 80 minutes by the injection of CZI in dosage of 1,000 units per Kg.

It is concluded from these studies that sequestration of insulin in peripheral tissue does not appear to protect the insulin from degradation. The ability of proteolytic enzymes to destroy insulin is not effected by the injection of 100 units of insulin per Kg. body weight.

Effect of Growth Hormone on Plasma Fatty Acids. M. S. RABEN * and C. H. HOLLENBERG, Boston, Mass.

Values of plasma unesterified fatty acids varied with fasting, feeding, glucose and insulin, as previously observed by others, in accordance with the concept that the value is an index of fat mobilization. Fasting values of normal and diabetic dogs regularly increased 100 per cent or more following partially purified porcine and purified

bovine growth hormone preparations. Growth hormone did not prevent the precipitous fall in concentration induced by glucose or glucose-insulin infusions, and differed from epinephrine which raised the value even under these circumstances. Growth hormone was effective for longer than 18 hours, in contrast to the brief action of epinephrine. Human growth hormone, prepared by the glacial acetic acid method and free of other pituitary hormones, raised fasting values in normal and hypopituitary human subjects. One and 2 mg. doses were effective in some normal adult subjects, and 4 mg. was regularly effective, doubling the value in four hours. No change occurred in this period without the hormone. A 4 mg. dose influenced the fatty acid concentration for at least 24 hours in hypopituitary subjects, but for shorter periods in normal individuals. Blood sugar concentration was not affected. Simian growth hormone was also effective but 30 mg. doses of equally purified porcine and bovine preparations were ineffective in this, as in other effects, in man. The findings suggest that growth hormone participates in the regulation of fat mobilization in man, and a physiological importance is inferred from the similarity of the dose promoting growth and increasing fatty acids. Two mg. of human growth hormone three times a week for 11 months to a pituitary dwarf produced a growth rate of 2.6 inches per year and raised the values of serum inorganic phorphorus and alkaline phosphatase.

Ventilatory Response to CO_2 in Patients with Congestive Heart Failure. LLOYD H. RAMSEY, JOHN GRISCOM, and JAMES SNELL, Nashville, Tenn. (Introduced by C. R. Park).

An increase in minute ventilation (V_E), often associated with the sensation of dyspnea, is a well documented occurrence in congestive heart failure. Since the hyperpnea is associated with low or low normal arterial CO_2 tension (Pa_{CO_2}) and normal oxygen saturation it is generally accepted that the hyperpnea is a result of reflex rather than chemical mechanisms. However, earlier studies indicate that such patients are even more sensitive than normal subjects to the inhalation of CO_2. This finding would indicate persistence of chemical control mechanisms and raise doubt concerning the singular importance of reflex mechanisms in the production of the hyperpnea. Unfortunately, these earlier studies which implied an increased sensitivity to CO_2 in heart failure are difficult to interpret because oxygen tensions were allowed to decrease simultaneously with the increase in CO_2.

Use of an open circuit method has allowed collection of V_E in normal and congestive failure subjects exposed to 2, 3 and 4 per cent CO_2 while keeping oxygen concentration at that of room air. Pa_{CO_2} was calculated from pH and CO_2 content of arterial blood.

The increase in V_E following 20 minutes' inhalation of each of the three CO_2 mixtures was considerably greater in the congestive failure group than in the normal subjects. This difference in response was greater in cardiac subjects with the higher resting V_E and although quite variable tended also to be greater in the patients with the lower Pa_{CO_2}.

Of considerable interest was the finding of a greatly increased ratio of physiologic dead space to alveolar ventilation (V_D/V_A) in the cardiac at rest which persisted at the higher levels of ventilation induced by CO_2. The V_A was only minimally increased at rest and the stepwise increase with CO_2 was not significantly different from the normal controls. Thus the difference in ventilation consisted almost entirely of dead space ventilation at rest and in response to CO_2. This increase in V_D can best be explained by a relatively low pulmonary blood flow in relation to ventilation. The work required for this dead space ventilation may well be a major factor in the production of the symptom of dyspnea.

The Effect of Isotonic Expansion of the Extracellular Fluid Volume on the Natriuretic Response to Infusions of Saline in Postoperative Patients. RUSSELL E. RANDALL, JR. and SOLOMON PAPPER, Boston, Mass. (Introduced by Maurice B. Strauss).

Since the "effective" extracellular fluid volume is a major determinant of renal sodium excretion in normal individuals, a study was devised to ascertain whether a decrease in this volume might be involved in the phenomenon of sodium retention commonly observed following major surgical procedures.

The renal excretion of sodium before and after the intravenous infusion of 2,000 ml. of 0.9 per cent sodium chloride solution over a 90 minute period was measured in three patients on the second to fourth day prior to a major orthopedic surgical procedure and on the second postoperative day. In each case the rate of sodium excretion *before* the challenging infusion was lower postoperatively than preoperatively. In two of the three cases the natriuretic response to the infusion postoperatively was only one-third of the preoperative response. That endogenous adrenal cortical activity is not involved in this phenomenon is suggested by the fact that a patient with Addison's disease maintained on a constant dose of hydrocortisone exhibited a similar response following cholecystectomy.

In three other patients, studied both before and after similar major orthopedic surgery, isotonic saline was infused in the postoperative study until the basal rate of sodium excretion increased to the preoperative level. At this time the 2,000 ml. challenging infusion was administered. The natriuretic response in each case was equal to or greater than that of the preoperative study.

These observations are consistent with the concept that a postoperative decrease in the "effective" extracellular fluid volume may be responsible for both sodium retention and the decreased natriuretic response to challenge following surgery.

An Enzyme in Plasma Inactivating Hageman Factor. OSCAR D. RATNOFF,* Cleveland, Ohio.

Blood circulating within the vascular tree is fluid; yet, when transferred to glass, clots readily. Earlier studies,

based on the work of Shafrir, deVries and Margolis, demonstrated that the clot-promoting effect of glass requires the presence of Hageman factor. Two types of inhibitory activity directed against Hageman factor may help maintain the fluidity of blood within its vessels. Previously, evidence was provided that glass freed Hageman factor from inhibition, initiating coagulation. Now experiments are described which suggest that plasma also contains an enzyme inactivating this freed Hageman factor.

Margolis reported that the clot-promoting factor in glass-treated plasma deteriorated rapidly; loss of activity seemed due to an antagonist in plasma. The deterioration of the clot-promoting factor in glass-treated plasma has been confirmed. The kinetics of inactivation of the clot-promoting property are enzymatic. Plasma also inactivates concentrated Hageman factor enzymatically, supporting evidence that Hageman factor is the clot-promoting factor in glass-treated plasma. The enzyme is present in normal plasma, 56° C.-heated plasma, delipidized plasma and plasma from patients deficient in Hageman factor. Its action is uninfluenced by glass or decalcification. The enzyme is concentrated in a fraction of plasma soluble successively at half-saturation with ammonium sulfate and upon dialysis at pH 5.2, ionic strength 0.02; it is found in Cohn fraction IV–4.

The following tentative hypothesis is suggested. The fluidity of blood is partly maintained by inhibitors directed against Hageman factor. Exposure of blood to glass converts Hageman factor to an active form, presumably releasing it from inhibition. The freed Hageman factor then initiates clotting. Once active, Hageman factor is enzymatically destroyed, preventing its continued action. The possible relationship between the formation of intravascular thrombi and the balance between the vascular surface and the inhibitors directed against Hageman factor remains to be defined.

Aminonucleoside Glomerulonephritis: Morphologic and Metabolic Studies. LILLIAN RECANT,* BENJAMIN A. BOROWSKY, and DAVID M. KESSNER, St. Louis, Mo.

Chronic glomerulonephritis has been induced in rats by administration of the aminonucleoside of puromycin (6-dimethyl-aminopurine-3-amino-D-ribose). Daily injections of 500 μg. per 100 Gm. rat for 50 to 70 days were required for development of the lesion. Morphologic changes in the kidney included hyalinization of the basement membranes of the glomeruli, proliferative changes in Bowman's capsule leading to crescent formation, and dilated tubules with protein casts. Previous observations indicated that larger doses of aminonucleoside given for two weeks induce a classical nephrotic syndrome in rats. Morphologic changes in such animals are minimal.

To obtain evidence of the mechanism of production of this experimental kidney disease, two types of studies were designed: *1*) determination of chemical configuration requisite for induction of the lesion, and *2*) effect of aminonucleoside on adenosine metabolism. In the first project, the following analogues of aminonucleoside were studied: 3-amino-D-ribose, 6-dimethyl-aminopurine, 6-dimethyl-adenosine, and 3-aminoadenosine. Despite the fact that these compounds contained one or the other basic moiety of aminonucleoside, none were found to produce nephrosis. The second study was prompted by the chemical similarity of aminonucleoside and the naturally occurring metabolite, adenosine. A yeast system known to produce acid labile phosphate (Po) in the presence of adenosine was utilized. With adenosine as substrate, 31.0 μM of Po was formed. The simultaneous addition of aminonucleoside and adenosine in molar ratios of 1:1 and 2:1 resulted in the formation of 34.5 μM and 6.8 μM of Po, respectively. When the concentration of aminonucleoside was raised to five times that of adenosine, complete inhibition of Po formation resulted. Paper chromatographic analysis and enzymatic assay identified the Po as adenosine triphosphate (ATP).

It is suggested that there exists a causal relationship between inhibition of ATP formation and the ability of aminonucleoside to induce experimental renal disease.

The Circulatory Dynamics in Pulmonary Emphysema During Treadmill Exercise. JOHN T. REEVES, ROBERT F. GROVER, GILES F. FILLEY, and S. GILBERT BLOUNT, JR.,* Denver, Colo.

To determine the effects of exertion on the cardiac output in emphysema, seven patients were studied by cardiac catheterization at rest and during exercise on the treadmill. All patients were evaluated clinically and by ventilatory, carbon monoxide and previous treadmill studies. Mild or severe diffuse obstructive emphysema was the primary or only disease present, and no patient had clinical heart failure at the time of study.

The results are compared to those in normal subjects and in other patients with emphysema, during rest and exercise, from this and other laboratories. In our patients the resting cardiac index was in the normal or low normal range. For the work performed and the oxygen taken up during exercise, the cardiac index was at least two S.D. below the mean normal in all but one instance, and in no patient was the index greater than normal. The increased demand for oxygen by the exercising tissues was met by a widening of the A-V difference. The mean exercise pulmonary artery pressures ranged from 36 to 105 mm. Hg, and the total pulmonary resistance ranged from 610 to 2,500 dynes-second-cm.$^{-5}$-M.^{2}. By determining the oxygen uptake, the oxygen content of arterial and mixed venous blood, and the pulmonary artery pressure at frequent intervals, it was found that a "steady state" existed in these patients after about five minutes of exercise.

It appears that in addition to disturbances in the transport of oxygen to the pulmonary capillary blood, these patients with emphysema show impairment in oxygen transport from the lungs to the tissues by virtue of an inadequate cardiac output during exercise.

A Study of the Adjustments to Sodium- and Water-Retaining Hormones in Normal Subjects. ARNOLD S.

RELMAN,* WILLIAM K. STEWART, and WILLIAM B. SCHWARTZ,* Boston, Mass.

In an attempt to clarify the mechanisms through which normal subjects "escape" from the effects of sodium- and water-retaining hormones, balance studies have been carried out in normals given desoxycorticosterone acetate (DCA) (20 mg. daily) or fluorohydrocortisone (5 to 10 mg. daily), with or without added vasopressin (ADH). Daily weights, blood pressure, sodium balance and endogenous creatinine and osmolar clearances were measured.

1. With intakes of 180 to 200 mEq. per day, urine sodium did not fall below 50 to 80 mEq. per day, even when the steroids were combined.

2. The phase of sodium retention lasted a few days and, after accumulation of 350 to 500 mEq., all subjects came into equilibrium or began "unloading," despite continued steroid. In only 2 of 17 experiments did mild hypertension appear, and in none was there overt edema.

3. Simultaneous DCA and ADH (10 units per day I.M.) did not enhance sodium retention; rather, "escape" occurred more promptly after only 70 to 200 mEq. sodium retention.

4. Continuous administration of DCA for 10 to 14 days while on a low sodium diet did not prevent the steroid from producing transient sodium retention when salt was then added to the diet. This was demonstrated by comparing the response to increased sodium intake with and without DCA.

5. In most experiments endogenous creatinine clearance tended to rise before or during "escape," but large changes in sodium excretion were sometimes unrelated to creatinine clearance.

It is concluded that: *a*) By themselves, hormones, even at very large doses, are not adequate to cause overt clinical edema and sodium-free urine. *b*) There is no evidence for the development of "resistance" to the tubular sodium-retaining effect of chronically administered DCA. *c*) While these observations suggest that an increase in GFR may be important in the "escape" from steroidal sodium retention, the present data do not permit any definitive evaluation of this factor.

Changes in Wedged Hepatic Venous Pressure and Hepatic Blood Flow Accompanying Clinical Improvement in Cirrhosis. TELFER B. REYNOLDS,* ALAN G. REDEKER, and HERMAN M. GELLER, Los Angeles, Calif.

To evaluate the natural history of portal hypertension, portal venous pressure has been assessed by recording wedged hepatic venous pressure (WHVP) during periods of apparent clinical improvement in patients with alcoholic cirrhosis. Nine patients were studied during their first episode of ascites and again from two to six months later when ascites had spontaneously disappeared. A rise in serum albumin accompanied the loss of ascites in each patient and icterus, present in four of them at the time of the initial study, subsided.

In three patients WHVP increased minimally (from a mean of 19.3 to a mean of 20.5 mm. Hg) despite the clinical improvement. Hemorrhage from esophageal varices occurred soon after the disappearance of ascites in each of these patients and in two instances was fatal.

In the remaining six patients significant falls in WHVP were noted at the time of apparent clinical improvement (from a mean of 15.5 to a mean of 9.8 mm. Hg). Radiologic evidence of esophageal varices has not appeared in any of these patients.

The fall in WHVP in six out of nine patients suggests that portal hypertension is not fixed and may decrease concomitantly with clinical improvement in cirrhosis. This observation may have some bearing on the problem of the "prophylactic" portacaval shunt.

The fall in WHVP was probably due to decreased hepatic vascular resistance since evidence of increased collateral flow around the liver did not develop and estimated hepatic blood flow, measured in four patients, increased from a mean of 1,200 ml. per minute to a mean of 1,435 ml. per minute.

Blood Sugar Regulation During Sleep in Normal and Diabetic Subjects. EUGENE D. ROBIN, DAVID M. TRAVIS, DESMOND G. JULIAN, CHARLES H. CRUMP, and BURIS BOSHELL, Boston, Mass. (Introduced by C. Sidney Burwell).

Ideal control of diabetes mellitus means that blood sugar levels of diabetics conform to diurnal curves of normals. Diurnal curves should include blood sugar changes occurring during sleep. Previous data on these changes are scanty. Therefore, blood sugar regulation during sleep was studied in normals and in diabetics.

Determinations of hourly blood sugar concentrations in normals during sleep reveal little variation from hour to hour. The greatest difference between hourly blood sugar concentrations in any subject was 26 mg. per cent. The mean standard deviation for the group was 5.3 mg. per cent. Results of glucose tolerance tests performed during the waking and sleeping state were essentially the same.

Thus, sleep is associated with an effective homeostatic mechanism for blood sugar regulation. However, blood sugar constancy does not depend on sleep *per se*. Studies of hourly blood sugar levels in nonsleeping normals reclining and fasted at night reveal the same constancy. This constancy is based on an effective regulating mechanism operating at a time when demands on this mechanism are minimal.

Diabetics are insulin deficient and lack an effective mechanism for blood sugar regulation. Wide variations in hourly blood sugars are found in both untreated and insulin-treated diabetics.

No untreated diabetic showed even approximately constant hourly blood sugar levels at night. Variations of as much as 91 mg. per cent were found. The mean standard deviation for the group was 19.4 mg. per cent.

Nor did insulin provide an effective regulating mechanism, since insulin-treated diabetics showed marked variations in hourly blood sugar levels.

Patterns of variation included hypoglycemia during

sleep, progressive hyperglycemia, and hyperglycemia for several hours followed by normoglycemia.

Insulin is a biochemical, but not a physiological, substitute for a sugar regulating mechanism. Ideal control necessitates consideration of the diabetic during sleep as well as during wakefulness.

Hormonal Influences on Intracellular Proteolytic Enzyme Activity. HERBERT G. ROSE, MARY C. ROBERTSON, and THEODORE B. SCHWARTZ,* Chicago, Ill.

A method has been devised for the assay of proteolytic enzyme activity in which cellular integrity is preserved. Since hormonal activity might, under some circumstances, require intact cells, such a system may help to resolve discrepancies that have arisen in substantiating the hypothesis that hormones act upon enzymes. The surviving rat diaphragm has been used as an enzyme preparation to hydrolyze the synthetic substrates glycylglycine and L-leucylglycine, thereby measuring dipeptidase and leucine aminopeptidase activities, respectively. Enzymatic behavior of this preparation is very similar to that of purified enzymes. A seasonal variation in enzyme activity was noted with a peak in summer and a fall in winter. In a number of situations enzyme activity changes occur in directions consistent with a functional role in the regulation of protein catabolism. Thus fasting increases aminopeptidase activity (controls, 43.8 ± 1.4 μM leucylglycine hydrolyzed per gram diaphragm per hour; fasted, 57.4 ± 2.1). Cortisone administration also increases activity (controls, 47.1 ± 2.1; injected, 60.6 ± 3.1), while adrenalectomy decreases activity (controls, 45.0 ± 2.5; adrenalectomized, 28.0 ± 1.2). Similarly, thyroidectomy reduces activity (controls, 57.2 ± 4.2; thyroidectomy, 39.8 ± 4.0), but only inconsistent increases could be obtained after triiodothyronine administration. In most instances, the changes observed also remained significant when referred to diaphragm dry weight, diaphragm nitrogen, and total diaphragm weight. It is concluded that hormonal and metabolic stimuli provoke changes in intracellular peptidase activity consonant with what would be predicted from studies conducted on a physiologic level.

Thiocyanate-Dischargeable Iodide in the Dog Thyroid Gland. I. N. ROSENBERG,* J. C. ATHANS, A. BEHAR, and C. B. TISCH, Boston, Mass.

Arterial and thyroid venous plasma concentrations of radioactive iodine were measured in acute experiments in the dog before and after the intravenous administration of thiocyanate or perchlorate. Radioactive iodine (50 to 300 microcuries I^{131}) was injected intravenously and two hours to seven days later, the animal being anesthetized with pentobarbital, one lobe of the thyroid was removed; the other was dissected so that its only visible remaining connections to the circulation were the vascular pedicle at the upper pole and the inferior thyroid vein, and a polyethylene catheter was inserted in the latter. Consecutive blood samples were then drawn from the inferior thyroid vein in heparin-coated syringes, while femoral arterial blood samples were obtained intermittently throughout the duration of the experiment (40 to 60 minutes). The plasma concentrations of total and of trichloroacetic acid-precipitable I^{131} were measured, and the acid-soluble I^{131} (presumably iodide) obtained by difference.

In experiments performed two to four hours after I^{131} administration, the accumulated glandular radioactivity was small and the arterio-thyroid venous iodide^{131} concentration difference was large; the arteriovenous difference was promptly abolished by thiocyanate administration, but only occasionally and transiently did the venous iodide^{131} concentration exceed the arterial. If thiourea was injected a few minutes before thiocyanate, the latter induced values of venous iodide^{131} concentration considerably larger than the arterial values. In experiments done three to seven days after I^{131} administration, glandular uptake was large, arteriovenous iodide^{131} differences were usually small, and perchlorate or thiocyanate administration led to a prompt and often sustained increase in the venous concentration of iodide^{131} so that the values were appreciably greater than the corresponding arterial values.

These results suggest the presence within the gland of inorganic iodide not newly entered from the plasma.

A Study of the Means by which Exercise Increases the Pulmonary Diffusing Capacity for Carbon Monoxide. JOSEPH C. ROSS, REGINA FRAYSER, and JOHN B. HICKAM,* Durham, N. C.

The diffusing capacity of the lung for O_2 and CO normally increases during exercise, as measured by various techniques. This is usually thought to be related to changes in the pulmonary circulation. Conceivably, exercise may increase diffusing capacity by *1*) effects secondary to increased cardiac output, such as enlargement of the pulmonary capillary bed, or *2*) effects secondary to increased ventilation, such as improving the distribution of blood and gas within the lungs. To separate these factors, measurements were made of the pulmonary diffusing capacity for CO by the "steady state" method of Filley (D_{co}) and the cardiac output by the dye method during procedures designed to alter pulmonary ventilation with minimal effects on output and to alter output and pulmonary vascular pressures with minimal effects on ventilation. Observations were made on normal, recumbent males.

In 7 subjects, leg exercise increased D_{co} from a resting mean of 30 to 49 ml. per mm. Hg per minute, cardiac output from 7.5 to 13.0 L. per minute, and ventilation from 8.5 to 22.2 L. per minute. In 7 subjects, the cardiac output was increased by drugs (epinephrine or norepinephrine and atropine) from a resting mean of 7.7 L. per minute to 13.3 L. per minute, but mean D_{co} and ventilation did not change. In 7 subjects, ventilation was voluntarily increased from a resting mean of 7.2 L. per minute to 21.8 L. per minute. There was negligible change in output, but D_{co} rose from the resting mean of 27 to 47 ml. per mm. Hg per minute. In 9 subjects, central venous pressure was increased by 5 to 10 mm. Hg with albumin infusion and G-suit inflation, but D_{co} did not in-

crease unless ventilation also increased. In a total of 67 simultaneous observations of D_{eo}, output, and ventilation in 24 subjects, D_{eo} and ventilation showed a highly significant positive correlation ($r = 0.0830$), but D_{eo} and output were not significantly correlated.

"Steady state" D_{eo} increases during exercise because ventilation increases; it appears insensitive to changes in output and pressure.

Systemic and Coronary Hemodynamics in Normal Men and Women. GEORGE G. ROWE, CESAR A. CASTILLO, GEORGE M. MAXWELL, D. J. FREEMAN, DOUGLAS H. WHITE, and CHARLES W. CRUMPTON,* Madison, Wis.

Systemic and coronary hemodynamics were determined by the Fick principle and N_2O methods, respectively, in 15 normal men and 15 normal women. Ages averaged 30 years for the men and 26 for the women but there was sufficient overlap in the groups that there was no significant age difference ($p < 0.1$).

Systemic hemodynamics were similar in that cardiac index and rate, right and left ventricular work index (LVWI), pulmonary and peripheral arterial pressures were not significantly different.

Coronary hemodynamics differed considerably. Coronary blood flow per 100 Gm. of left ventricular myocardium per minute (CBF) was 26.5 per cent greater in the women ($p < 0.001$) and their coronary vascular resistance was 35 per cent less ($p < 0.01$). Myocardial oxygen consumption ($CMRO_2$) and carbon dioxide liberation were not significantly different. Although the hemoglobin and hence the arterial (AO_2) and mixed venous oxygen content were less in the women ($p < 0.001$), the product of CBF times AO_2 was greater (+16.6 per cent, $p < 0.05$) indicating that more O_2 was available to the female heart. Females also had more CBF per kilogram meter of LVWI (+26 per cent, $p < 0.01$) and a reduced LVWI to $CMRO_2$ ratio (−28 per cent, $p < 0.05$).

This study may be interpreted to mean that the heart of women is better perfused by blood than the heart of man and perhaps this is associated with the known lower incidences of coronary artery disease in females.

Chronic Salivary Gland Virus Infection in Children. WALLACE P. ROWE, Bethesda, Md. (Introduced by Harry Eagle).

Salivary gland virus is known to cause cytomegalic inclusion disease of the newborn, but infection in older individuals is poorly understood. Serological studies indicate that infection is highly prevalent. Using tissue culture techniques for serological and virus isolation studies, a survey was made of infection in lower economic group children, aged four months to four years. It was found that 18 per cent of children in the Washington, D. C., area had antibodies, while in other countries the proportion of positives reached 60 to 80 per cent. Four per cent of clinic children had virus in their saliva as determined by a single test. Since virus was found only in children with complement fixing antibody, a longitudinal study was made of antibody positive children in a receiving home nursery. When repeated tests were made, virus was recovered from the saliva on one or more occasions from two-thirds of the children with antibody. Twelve children excreted virus for more than a month, and several were demonstrated to excrete virus over periods of at least five to eight months. On the basis of antibody studies, some of the virus positive children had been infected for at least 12 to 24 months. Ten of 11 children who were excreting virus in their saliva also had demonstrable virus in their urine, although inclusion cells were not found in repeated smears of the urinary sediment of 7. Careful clinical studies of 16 oral carriers showed a variety of abnormalities, mostly of a nondescript nature. The only finding which appeared to be in excess of the abnormalities in other children in the same population was white cells or casts in the urinary sediment of 6, including two cases with marked elevation of Addis counts.

Intestinal Similarities Between Celiac Disease and Idiopathic Sprue. CYRUS E. RUBIN, LLOYD L. BRANDBORG, PATRICIA C. PHELPS, and HAWLEY C. TAYLOR, JR., Seattle, Wash. (Introduced by Robert H. Williams).

To investigate the possible relationship between celiac disease and idiopathic sprue, 110 suction biopsies of the small bowel were performed in 65 patients comprising idiopathic sprue, suitably aged controls, and active, latent, and adult celiac disease with well established diagnoses.

A flexible tube, 4 mm. in diameter, was designed for safe biopsy in infants and adults. Under fluoroscopic control a mucosal knuckle was excised with measured negative pressure. The gross specimen's absorptive surface and vasculature were photographed and examined microscopically during fixation. In serial sections the percentage of epithelial cells observed in a total count of epithelial cells plus a band of muscularis was estimated by a modified Chalkley technique employing a mold-counting micrometer grid.

The surface in normal controls (adults, 25; children, 14) was covered with well vascularized slender villi. In 10 sprues, 2 of 3 adult, 1 of 9 juvenile, and 1 of 4 "cured" celiacs, villi were absent, vessels reduced in number and the surface flattened and knobby. Steatorrhea uniformly accompanied the latter findings. Histologically there was loss of villi, altered surface epithelium, chronic inflammatory infiltration, and crypt abnormalities suggesting retardation of mucosal replacement. A varied spectrum from normal to moderately severe mucosal alterations was evident in the remaining celiacs: adults, 1 moderate; "cured," 2 normal, 1 moderate; active, 2 mild, 6 moderate.

In sections there was a marked reduction from the normal length of absorptive epithelium in severe lesions. To test this visual impression the modified Chalkley counts described above were blindly made by a nonclinical observer. The difference between normal and severely abnormal groups was highly significant statistically by a chi-square test, with $p < 0.0001$.

The similarity of the intestinal lesions in idiopathic sprue and celiac disease suggests that they are the same

process. More prolonged observations will test this theory.

Transcortin: A Corticosteroid-Binding Plasma Protein. AVERY A. SANDBERG and W. ROY SLAUNWHITE, JR., Buffalo, N. Y. (Introduced by Frank H. Tyler).

Previous work in our laboratory indicated that human serum albumin (HSA) accounts for the preponderant part of the binding of estrone, estradiol, progesterone and testosterone in the blood. Cortisol and corticosterone were bound to plasma to a much greater extent, however, than they were to HSA. This indicates the presence of a protein(s), with great avidity for cortisol and corticosterone. By analogy to transferrin, we designate it as transcortin. The present studies are based on experiments utilizing equilibrium dialysis of 1 per cent HSA containing C^{14}-labeled and carrier steroid against 1:5 dilutions of plasma in saline. The results indicate that 75 to 90 per cent of tracer doses of cortisol and 60 to 75 per cent of corticosterone are bound by transcortin under these conditions. Undiluted plasma bound 100 per cent of tracer amounts of cortisol. The binding capacity of plasma transcortin was measured by adding increasing amounts of carrier cortisol to the HSA and was shown to be approximately 20 μg. per 100 ml. of undiluted plasma. Thus, transcortin would seem to be nearly saturated at normal plasma levels of cortisol. Testosterone, progesterone and 11β-hydroxyandrostenedione showed little (12 to 32 per cent) and estrogens no binding to plasma proteins other than HSA.

An hypothesis can therefore be formulated regarding the significance of changes of transcortin concentration. If one assumes that transcortin-bound cortisol is inactive, the pharmacological and physicological effects of cortisol may be a function of the steroid not bound by transcortin. Thus, reduction of transcortin concentration may account in some measure for manifestations in patients with Cushing's disease having apparently normal adrenals; and increased levels of transcortin for the high levels of cortisol in pregnancy without manifestations of hyperadrenocorticism. The development of methods for measuring transcortin concentration will shed light on this hypothesis.

An Explanation for and Experimental Correction of the Abnormal Water Retention in Cirrhosis. HAROLD P. SCHEDL and FREDERIC C. BARTTER,* Bethesda, Md.

Diuresis was induced in patients with hepatic cirrhosis and in normal subjects with isotonic dextrose given intravenously. That the impaired water diuresis of cirrhotics was *not* due to excessive or persistent antidiuretic hormone (ADH) was confirmed by the observations that *1*) urine tonicity fell readily below that of serum, *2*) physiologic amounts of administered vasopressin (Pitressin®) could prevent this fall, and *3*) administered Pitressin® had only normal duration of action.

If "free" water is generated by tubular reabsorption of sodium, the minimal free water clearance (C_{FH_2O}) and resultant tendency to hyponatremia of cirrhotics is paradoxical. Their almost quantitative reabsorption of filtered sodium would imply *greater* than normal C_{FH_2O}. Excessive proximal reabsorption of glomerular filtrate in these patients could prevent diuresis by withholding sodium and water from distal sites where free water could be generated.

These hypotheses were tested by repeating the infusions with volume kept constant, but with progressively greater amounts of mannitol substituted for dextrose. With increasing mannitol proportions, normals showed the expected progressive fall in C_{FH_2O}, which reached negative values with isotonic mannitol. In contrast, cirrhotics showed increases in C_{FH_2O} up to seven times that with isotonic dextrose; even when only isotonic mannitol (and thus no free water) was given, C_{FH_2O} was as much as twice that with isotonic dextrose.

As further evidence for increased cation transfer at distal tubular sites, potassium excretion increased with mannitol in cirrhotics, but not in normals.

These findings support the concept that retention of water loads and hyponatremia in cirrhotics result not from ADH but from excessive proximal reabsorption of sodium and water. Delivery of a sufficient volume of glomerular filtrate from proximal to distal sites with "osmotic" diuretics produces more free water in cirrhotics than in normals under identical conditions.

ECHO-9 Virus Exanthema. WILLIAM F. SCHERER, JAMES PRINCE, and JOSEPH ST. GEME, Minneapolis, Minn. (Introduced by Jerome T. Syverton).

During the summer of 1957, an epidemic of exanthema became manifest in Minnesota. Patients presented fever and a generalized morbilliform rash which lasted from one to three days. Family infections and spread within neighborhoods were common. Although rash was the only symptom in many cases, a spectrum of disease was displayed which ranged from inapparent asymptomatic infection to skin rash with little disability, to skin rash and fever, to skin rash and aseptic meningitis, and finally, to aseptic meningitis without rash. In an attempt to determine the etiology of this syndrome, specimens from a selected group of patients were studied by tissue culture techniques.

In 21 patients ECHO-9 virus neutralizing antibodies appeared during convalescence and in 15 of the 21 patients ECHO-9 virus was recovered from throat swabs, feces and/or cerebrospinal fluids. The Minnesota strain of ECHO-9 virus was cytopathogenic for human foreskin fibroblasts; it differed from the prototypic strain of ECHO-9 virus and resembled the European, English and Canadian strains in its pathogenicity for newborn mice.

Compensatory Mechanisms in Primaquine-Sensitive Erythrocytes. STANLEY L. SCHRIER and ROBERT W. KELLERMEYER, Chicago, Ill. (Introduced by Alf S. Alving).

"Primaquine-type" drug-induced hemolytic anemia occurs in otherwise healthy Negroes, and rarely in Cauca-

sians. While drug-sensitive erythrocytes are normal morphologically and immunologically, they undergo acute intravascular hemolysis when exposed *in vivo* to primaquine, sulfanilamide, nitrofurantoin (Furadantin®), furazolidone (Furoxone®), other drugs and fava beans. Characteristically these erythrocytes have a normal oxidized glutathione content but a low, unstable reduced glutathione content. We believe that the primary enzymic abnormality is a genetically determined deficiency of glucose-6-phosphate dehydrogenase, with compensatory increases in glutathione reductase and aldolase. Erythrocytes deficient in glucose-6-phosphate dehydrogenase might be deprived of energy resulting from triphosphopyridine nucleotide (TPN) reduction in the oxidative hexosemonophosphate shunt, and, therefore, obliged to derive energy from diphosphopyridine nucleotide (DPN) reduction.

Aldolase and glyceraldehyde-phosphate dehydrogenase provide substrate for, and catalyze, the initial oxidative phosphorylation in anaerobic glycolysis (Embden-Meyerhof pathway), providing energy in the form of reduced DPN and high energy phosphate bonds. These enzymes were assayed in hemolysates by modifications of the Warburg and Christian method.

Aldolase was increased in hemolysates from 10 primaquine-sensitive individuals when compared with 13 normals, independent of variations in the following reaction conditions: time, amount of hemolysate, temperature, pH, substrate concentration, aerobiosis and anaerobiosis. In contrast, glyceraldehyde-phosphate dehydrogenase in hemolysates of primaquine-sensitive erythrocytes was normal.

Glyceraldehyde-phosphate dehydrogenase has been called a "pacemaker of anaerobic glycolysis." Its substrate, triosephosphate, does not accumulate in erythrocytes, while the substrate for the preceding reaction, fructose-d.phosphate, is present in considerable amounts. We propose that increased aldolase could convert fructose-diphosphate to additional triosephosphate, which glyceraldehyde-phosphate dehydrogenase could use to reduce DPN. Thus erythrocytes deficient in glucose-6-phosphate dehydrogenase theoretically could utilize energy sources of anaerobic glycolysis more effectively and maintain normal life spans unless challenged by hemolytic drug.

The Renal Response to Acetazolamide (Diamox®) During the Metabolic Acidosis of Salicylate Intoxication. ROBERT SCHWARTZ, FRANCIS X. FELLERS, JOHN KNAPP, and SUMNER YAFFE, Boston, Mass. (Introduced by Charles A. Janeway).

These studies were initiated to determine the extent to which alkalinization of urine could be achieved rapidly (since salicylate excretion is a function of urinary pH) without further compromising acid-base homeostasis during metabolic acidosis.

Three infants with salicylate levels greater than 25 mg. per cent and with advanced metabolic acidosis were investigated for the effects of salicylate intoxication on the excretion of individual cations (Na, K, Ca, Mg, and NH_4), anions (organic acids, HCO_3, Cl, phosphate) urinary pH, and salicylates. During control observations while sodium bicarbonate was infused, glomerular filtration rate (GFR) (creatinine or inulin) was depressed to half of normal in two infants and renal plasma flow (PAH) was depressed in two. Control urine pH's ranged from 5.07 to 5.54 and less than 2 per cent of the filtered nonbound salicylate was excreted. Although ammonium and titrable acid resulted in buffer conservation of 101 to 146 μEq. per minute per M.^{2}, organic acids (ketones) (174 to 512 μEq. per minute per M.^{2}) contributed significantly to net buffer cation loss.

Diamox® (5 to 10 mg. per Kg.) was given intravenously after several control periods and while bicarbonate infusion continued. The latter prevented progression of the acidosis and in most cases the serum CO_2 content actually increased. With an increase in urine flow and urinary pH (maximally 7.21 to 7.70), salicylate excretion increased markedly from excretion/filtration (E/F) less than 0.02 to maximum E/F 0.28, 0.64, 0.83. Salicylurate and other salicylate conjugates were not affected. Ammonium and titrable acidity were diminished, while Na and particularly K increased. Although filtered bicarbonate was low (less than 10 mEq. per L.) and GFR depressed, bicarbonate excretion increased to as much as 30 per cent of filtered load. Organic acid excretion remained elevated throughout and buffer cation loss (organic acid plus bicarbonate) after Diamox® was two to three times the control values.

The variability in buffer cation loss after blocking the major renal compensatory mechanism precludes reasonable prediction of buffer needs to maintain homeostasis.

The Kinetics of Renal Bicarbonate Reabsorption During Acute Respiratory Acidosis. WILLIAM B. SCHWARTZ,* ADRIEN FALBRIARD, and GUY LEMIEUX, Boston, Mass.

The kinetics of renal bicarbonate reabsorption have been studied during acute sustained respiratory acidosis (pCO_2 80 to 110 mm. Hg) in anesthetized dogs whose plasma bicarbonate levels were elevated by infusion of sodium bicarbonate. With progressive elevation of plasma bicarbonate concentration, there was a curvilinear rise in the rate of reabsorption which at the highest plasma levels studied (55 to 60 mEq. per L.) reached an average level of 3.8 mEq. per 100 ml. glomerular filtration (GFR). Frank excretion of bicarbonate began at plasma levels of 26 to 30 mEq. per L. The data indicate that elevation of pCO_2 does not simply increase the reabsorptive capacity to a single new constant rate, but rather suggests that during acute sustained respiratory acidosis the reabsorption of bicarbonate is a function of plasma bicarbonate concentration.

The pattern of bicarbonate reabsorption is similar to that which has been observed previously with partial inhibition of carbonic anhydrase in animals with metabolic acidosis. Analysis of these latter data in terms of Michaelis-Menten theory yielded findings consistent with the hypothesis that enzyme activity had become rate-limiting in the reabsorptive process.

The present data were plotted in a similar fashion according to the Lineweaver-Burk modification of the Michaelis-Menten equation on the assumption that filtrate bicarbonate concentration was equal to (or approximated) some constant and direct function of S for the overall reabsorptive process and that reabsorption per 100 ml. GFR was equal to v. The fit of the data with Michaelis-Menten kinetics was consistent with the hypothesis that in acute respiratory acidosis carbonic anhydrase becomes rate-limiting in the reabsorptive process, and this interpretation provides a tentative explanation for the increasing rate of bicarbonate reabsorption seen with elevation of the plasma bicarbonate level. As a corollary, the slower and constant reabsorptive rate, independent of plasma bicarbonate concentration, which is seen with normal pCO_2 would appear to be directly determined by carbon dioxide tension which seems to prevent full utilization of enzyme capacity.

A Model for the Appraisal of Glucose Metabolism. J. C. SEED, F. S. ACTON, and A. J. STUNKARD, New York, N. Y., Princeton, N. J., and Philadelphia, Pa. (Introduced by F. D. W. Lukens).

The interpretation and correlation of data on carbohydrate metabolism can be simplified by plotting separately the behavior of the major factors concerned with glucose homeostasis. The combination of such plots gives a reasonably complete description of glucose homeostasis.

Currently available data permit one to plot as a series of straight lines, the major components of the system mediating glucose homeostasis. Such straight lines, representing a series of linear differential equations, were plotted as follows: *1*) glucose utilization (dx/dt) against glucose concentration (x) for liver, muscle (periphery) and brain, *2*) glucose production by the liver, and *3*) the rate of glucose diffusion in a two compartment system. Inspection of such plots, which are based on experimental data, allow the prediction of changes in any element of the system when another element is altered, as for example, by a glucose load, the administration of insulin, or muscular exercise. Results of this inspection may be given more precise form by combining and integrating the differential equations by the techniques of numerical calculus.

The equation obtained by this integration has already proven useful in clinical work. It has greatly facilitated interpretation of the intravenous glucose tolerance test. Comparison of the integrated equation to the raw data of the test permits the calculation of the values for the individual physiological functions which are required to produce the final shape of the curve. The equation also accounts for the hypoglycemia occurring during the intravenous glucose tolerance test and for the fact that this hypoglycemia is proportional to the rate of fall of blood glucose concentration.

The Biosynthesis and Metabolism of L-Fucose in Man. STANTON SEGAL and YALE J. TOPPER, Bethesda, Md. (Introduced by Truman Schnabel, Jr.).

A significant amount of L-fucose (6-deoxy-L-galactose) is known to occur in man, not only as a constituent of blood group polysaccharides in red blood cells, saliva, gastric mucin and ovarian cysts, but also as the free sugar in urine and milk. This sugar is of interest physiologically because of its role in blood group specificity and biochemically because of its unusual structure. To study fucose biosynthesis, glucose-6-C^{14} was administered intravenously to two normal postpartum lactating females. Milk was collected and pooled five hours later. Fucose and lactose were isolated and chemically degraded. The pattern of C^{14} labeling in fucose was found to be similar to that in the other hexoses, suggesting the direct conversion of glucose to fucose with the hexose carbon skeleton remaining intact. Such a transformation involves the reduction of carbon atom 6 and the inversion of the configuration of C-2, C-3 and C-5. Metabolism of fucose was studied by the intravenous administration of fucose-1-C^{14} to a normal subject. Within six hours 57 per cent of retained C^{14} was excreted as expired $C^{14}O_2$, peak labeling of $C^{14}O_2$ being observed within 30 minutes. Thirty-two per cent of the C^{14} dose was excreted in the urine, mostly as unaltered fucose. *In vitro* studies to delineate the pathway of fucose catabolism in mammalian tissues are being undertaken with rabbit and beef tissue. Fucose appears to be metabolized to CO_2 chiefly by liver and kidney, with very little catabolism occurring in brain, skeletal muscle, spleen, or red blood cells. These studies have demonstrated that despite its unusual configuration, L-fucose is both synthesized and rapidly metabolized by man.

Relationships of Hypertension and Renal Impairment to Experimental Chronic Pyelonephritis in Rats. ALVIN P. SHAPIRO, Pittsburgh, Pa. (Introduced by I. Arthur Mirsky).

Chronic pyelonephritis was produced in over 100 rats by kidney massage and intracardiac inoculation of bacteria, as previously described. Animals infected repeatedly with *Escherichia coli, Proteus morgagni,* and *Streptococcus zymogenes,* were followed as long as one year, and developed predominantly tubular lesions while glomeruli were relatively intact. Excretory function was significantly impaired. Average osmolal concentration after 24 hours of dehydration was 1,300 milliosmols per Kg. (normal, 2,100 milliosmols per Kg.). Azotemia was absent until osmolality fell below 60 per cent of normal. Despite these changes, vascular disease and significant hypertension did not develop.

Chronic pyelonephritis is considered an important cause of hypertension in man, although many patients with the disease remain normotensive. Absence of hypertension in experimental chronic pyelonephritis, despite otherwise typical pathologic and functional changes, suggested increased susceptibility to renal infection as an alternative explanation for the clinical association between pyelonephritis and hypertension. This possibility has been studied as follows:

Inoculation of *E. coli, without renal massage,* produced

no gross pathologic lesions in kidneys of 15 normotensive rats and positive renal cultures in 20 per cent with only one culture demonstrating heavier than 1 plus growth. This result duplicated previous studies in which the necessity of kidney massage for production of *E. coli* pyelonephritis in normal rats was established. On the other hand, in 17 rats made hypertensive with desoxycorticosterone acetate (DCA) and salt, inoculation of *E. coli, without massage,* resulted in gross lesions in 30 per cent of the kidneys, and positive cultures in 38 per cent, three-fourths of these showing heavier than 1 plus growth. With massage, gross lesions after *E. coli* inoculation occurred in 54 per cent of normotensive and 75 per cent of hypertensive kidneys.

Chronic pyelonephritis in rats produces renal lesions which are functionally and pathologically tubular in character, but hypertensive disease does not result. The data suggest that the relationship between chronic pyelonephritis and hypertension may at least partially represent increased susceptibility of the hypertensive kidney to pyelonephritis.

Hemadsorption in the Diagnosis and Study of Viral Infections. ALEXIS SHELOKOV, Bethesda, Md. (Introduced by Joseph E. Smadel).

Erythrocytes of certain animal species adsorb onto the surface of monolayer tissue cultures infected with the hemagglutinating viruses. The reaction is inhibited by specific antibody and can be used in neutralization tests for serologic diagnosis and virus identification.

Hemadsorption technique, employing monkey kidney cell cultures and guinea pig erythrocytes, applied to 93 throat washings from patients suspected of influenza, yielded 21 strains of Asian virus. Moreover, in some instances new isolates were recognized and definitively typed within two days after initiating testing. Only 10 strains were recovered from these specimens when the usual chick embryo procedure was used.

In addition to influenza viruses, hemadsorption is associated with the following: mumps, CA (croup-associated), vaccinia and two newly discovered agents recovered from children with respiratory disease which are designated "hemadsorption viruses 1 and 2." Polioviruses, ECHO viruses types 1 through 11, adenoviruses types 1, 2, 4, 6, 7 and 8, Coxsackie viruses A9, B-1 through B-5, and herpes simplex virus do not display hemadsorption.

Hemadsorption provides a simple, rapid means for diagnosing and studying a number of viral diseases.

A Study of the Transplacental Transfer of Thyroid Hormones in Sheep. ROBERT H. SHEPPARD, V. MARILYN TURNER, and WILLIAM M. PAUL, Toronto, Canada. (Introduced by W. Hurst Brown).

Evidence for or against the passage of thyroid hormones across the placenta is conflicting. Most studies conducted to date have been indirect, *e.g.,* attempting with thyroid hormone to prevent the formation of thiouracil or percholate fetal goiters. When I^{131} labeled hormones have been used, samples were usually too small or activity too low for adequate chromatography or autoradiography.

It is possible, in the sheep, to place a cannula directly into a sufficiently large fetal artery that adequate blood samples can be withdrawn without interfering with the normal placental circulation, and without leakage. This was done in a series of 15 experiments and radioactive iodine (I^{131}) alone or I^{131} labeled L-thyroxine or L-triiodothyronine was administered on different occasions to mother or to fetus. Serial blood samples were taken from each circulation up to six hours from the time of injection. Utilizing an ethanol extraction technique and paper chromatography in a butanol-dioxane-ammonia solvent system, L-thyroxine, L-triiodothyronine and iodide were separated. The radioactivity was measured by cutting each chromatogram into a series of transverse strips and by counting each strip in a well-type scintillation counter. The peaks of radioactivity were identified by the use of suitable added standards.

Almost immediately, I^{131} administered as sodium iodide passed across the placenta. After administering labeled hormone, some radioactivity could be detected in the opposite circulation. However, on chromatography, all of this activity was found to be in the iodide area and was presumably the result of hormone deiodination. None was present in the L-thyroxine or L-triiodothyronine areas. Thus, in these acute experiments, no transmission of labeled thyroid hormones from mother to fetus or from fetus to mother was demonstrated.

The Appearance of a Fibrinolysin Activator in the Blood of Patients with Enhanced Fibrinolytic Activity. SOL SHERRY,* ROBERT I. LINDEMEYER, ANTHONY P. FLETCHER, and NORMA ALKJAERSIG, St. Louis, Mo.

Increased fibrinolytic activity in the human circulation has been reported following electroshock therapy, pyrogen injections, intense exercise, epinephrine, and ischemia. The mechanism underlying this increased activity has not been investigated. Two possible explanations have been advanced: a reduction in the antifibrinolytic activity of blood, or the appearance in the blood of an activator of the naturally occurring profibrinolysin.

Utilizing a variety of methods, a study has been made of the state of fibrinolytic activity in the blood of patients subjected to the various stresses mentioned above. The lysis time of a clot formed from the plasma euglobulin fraction and the ability of the plasma euglobulin to lyse preformed I^{131} tagged human plasma clots proved to be most sensitive for demonstrating the appearance of increased fibrinolytic activity.

Increased fibrinolytic activity could be demonstrated in almost all patients subjected to electroshock, pyrogen administration, or intense exercise, but was observed with less consistency following epinephrine and local ischemia. With the latter, fibrinolytic activity, when observed, was most striking in the venous blood draining the ischemic area, confirming the recent observations of Kwaan that fibrinolytic activity can be locally produced.

The production of intense fibrinolytic activity in the blood of these patients was associated with the appearance of a fibrinolysin activator and without significant reduction in antifibrinolytic activity, profibrinolysin or fibrinogen concentration. The presence of an activator was demonstrated by activation experiments employing purified profibrinolysin as the substrate and the plasma euglobulin as the source of activator. These observations support the view that under stress situations of the type described, circulating fibrinolytic activity increases due to the release of a fibrinolysin activator.

Inhibition of Water Reabsorption in the Intact Perfused Renal Tubule by Ouabain and Dinitrophenol: Evidence for Active Sodium Transport. JOSEPH C. SHIPP, Boston, Mass. (Introduced by Eugene Eppinger).

The following experiments were designed to study water and sodium reabsorption by the renal tubule. The effects of ouabain and dinitrophenol on water reabsorption were studied. Ouabain, a cardiac glycoside, is known to inhibit cation transport in isolated tissues. Dinitrophenol reduces the available metabolic energy required for active transport.

Collection and stopped flow perfusion experiments were performed on the intact proximal renal tubule of anesthetized Necturus. Classical micropuncture techniques of Richards were used. C^{14} labeled inulin served as an indicator of net water movement. In stopped flow perfusions a tubule was blocked with oil near the glomerulus and distally; the tubule was filled and the perfusate removed after 20 minutes.

In six experiments, fluid collected from the distal 20 per cent of the proximal tubule showed a tubular fluid ÷ serum inulin ratio of 1.52, indicating a mean water reabsorption of 33 per cent. Similar collections in four animals which had received ouabain (0.01 mg. per animal) intravenously showed a decrease in net water reabsorption to 15 per cent. In control studies stopped flow perfusions showed a mean water reabsorption of 28 per cent, in good agreement with the collection data. In seven perfusion experiments, ouabain in the perfusate (1.4×10^{-4} M per L.) reduced net water reabsorption to 10.2 per cent, indicating direct inhibition of the mechanism responsible for water reabsorption. This direct action on tubular transport may contribute to the diuretic effect of cardiac glycosides in man. In seven experiments where dinitrophenol (2×10^{-4} M per L.) was included in the perfusate, water reabsorption was reduced to 9.8 per cent. This suggests that water transport is effected by a mechanism whose energy supply is susceptible to dinitrophenol inhibition.

The present observations of water movement in a renal tubule known to have a transtubular electrical potential, negative on the luminal side, are consistent with the hypothesis that active Na transport is responsible for water reabsorption.

Thyroid Secretion of Nonthyroxine Iodine. D. W. SLINGERLAND, E. S. DELL, D. E. GRAHAM, A. P. TRAKAS, and B. A. BURROWS,* Boston, Mass.

Measurements were made in hyperthyroid patients and euthyroid subjects of thyroidal radioiodine, serum protein-bound radioidine (PBI^{131}) and chemical iodine (PBI^{127}) concentrations and urinary radioiodine excretion (URI^{131}) following a tracer I^{131} (500 μc). After five to seven days of equilibration the administration of methimazole (Tapazole®) resulted in a rapid increase in URI^{131}, predictable from a complete block of thyroid uptake of "recycled" iodide in hyperthyroidism, but greater than the predicted value in normal subjects.

Usually, during the course of Tapazole®, the ratio of daily URI^{131} to PBI^{131} concentration gradually diminished. In some patients this ratio reached a constant value, although PBI^{127} continued to fall. In several patients this ratio decreased abruptly by as much as 60 per cent with the administration of iodide, with a slowing of the apparent discharge of thyroidal radioiodine, but without appreciable changes in PBI^{131} or PBI^{127}. If the later urinary radioiodine is derived solely from the peripheral degradation of PBI^{131}, then the earlier ratio (significantly higher than the final ratio) of URI^{131} must represent iodine from some source other than circulating hormonal radioiodine.

These findings were confirmed by repeated short-term studies with I^{132}: Serum inorganic I^{127} and I^{131} (calculated from serum I^{132} levels and urinary iodine specific activities) increased sharply with Tapazole® and the serum inorganic I^{131} fell with iodide administration. Studies of I^{131}-thyroxine degradation in myxedema did not reveal any peripheral effects of Tapazole®, iodides, or triiodothyronine.

This study suggests that iodide or organic iodine, other than thyroxine, is being secreted from the thyroid gland of hyperthyroid patients in significant amounts; that antithyroid compounds (including iodide) may suppress the release of this material; that antithyroid compounds may block the intrathyroidal cycling of iodide released by deiodinating mechanisms in normal subjects; and that a defect in reutilization of such intrathyroidal iodine may exist in untreated hyperthyroidism.

Pyrimidine Metabolism in Man: The Biosynthesis of Orotic Acid. LLOYD H. SMITH, JR. and FAITH BAKER, Boston, Mass. (Introduced by Edward Bland).

Considerable information is available concerning purine metabolism in man. This has been obtained primarily by the study of uric acid excretion and the rate of incorporation of isotopic precursors into it. In contrast almost no information is available concerning pyrimidine metabolism in man, due in large measure to the absence of a pyrimidine excretory product comparable to uric acid. The potential importance of methods for assaying pyrimidine metabolism in man is apparent from a consideration of the genetic and controlling functions of nucleic acids, the role of pyrimidine nucleotides in intermediary metabolism, and the increasing interest in pyrimidine analogues as anti-neoplastic agents.

In this overall study of pyrimidine metabolism, initial attention has been directed to the synthetic pathway. Using C^{14}-labeled precursors the following enzymatic steps

in pyrimidine synthesis have been identified in circulating human blood cells:

I Carbamylphosphate + aspartate $\rightleftharpoons$ carbamylaspartate.

II Carbamylaspartate $\overset{-H_2O}{\rightleftharpoons}$ dihydroorotate.

III Dihydroorotate $\overset{-2H}{\rightleftharpoons}$ orotate.

The enzymes involved in I and II are found in solution in red cell hemolysates and white cell sonicates, in which their biochemical characteristics have been determined. The enzyme of III has not been obtained in solution, has been found to be localized in white cell nuclei, and is virtually absent from circulating erythrocytes. Step III can be demonstrated, however, in nucleated avian erythrocytes. For each step the normal range of enzyme activity per unit number of cells has been determined. Free carbamylaspartate, dihydroorotate, and orotate could not be demonstrated in plasma or erythrocytes. The usefulness of these assay systems for the *in vitro* evaluation of a newly synthesized inhibitor of pyrimidine formation, *l*-carbamyl cysteic acid, has been demonstrated. These studies are currently being extended to leukemic cells.

Metabolism of Cortisone by Malignant Prostatic Tissue. JOSEPH E. SOKAL and W. ROY SLAUNWHITE, JR., Buffalo, N. Y. (Introduced by Philip K. Bondy).

When patients with advanced carcinoma of the prostate were given 300 mg. of cortisone acetate per day, a striking rise in their total urinary 17-ketosteroid output was observed; this phenomenon did not occur in normal men or in patients with debilitating non-neoplastic diseases (Sokal *et al.*, Yale J. Biol. Med. 1953, **25**, 320). This finding has been confirmed in an additional group of patients. This effect was not observed in most patients with other cancers, but was seen in some. Next, extraction and fractionation of the 17-ketosteroids excreted during baseline and cortisone periods was accomplished in three consecutive patients with carcinoma of the prostate. It was found that the difference between these patients and controls is due principally to a rise in 11-ketoetiocholanolone excretion, which averaged 14.3 mg. per 24 hours more than the baseline excretion, as compared with 2.0 mg. per 24 hours for controls with cancer of the lung.

In order to determine whether this phenomenon represents a host change in patients with prostatic carcinoma or is a specific manifestation of the metabolic activity of the tumor, slices of prostatic adenocarcinoma obtained at surgery were incubated with radioactive cortisone *in vitro*. The conversion of cortisone to adrenosterone was demonstrated in both of two experiments. Presumably this compound is further metabolized, probably in the liver, to the 17-ketosteroids identified in the urine. Thus the abnormal urinary ketosteroid pattern observed in patients with prostatic carcinoma during cortisone administration reflects a hitherto unsuspected metabolic activity of this tumor. The conversion observed in these experiments has not previously been reported for human tissue *in vitro*.

Studies of Properdin in Cancer Patients. CHESTER M. SOUTHAM, New York, N. Y. (Introduced by J. H. Burchenal).

A survey of serum properdin levels in over 150 patients with neoplastic diseases of many types confirms the previously reported increased frequency of low properdin levels (less than 2 units per ml. by Pillemer's zymosan technique). The total data, as well as serial determinations in individual cancer patients, indicate that properdin depression tends to correlate with severity of disease, but several unexplained discrepancies have been found.

Sera from several cancer patients gave a zone effect in successive serum dilutions in the zymosan assay. This may be caused by properdin inhibitors, which have been demonstrated in two sera, or by a high titer of endogenous complement (C'3) which obscures the properdin-zymosan reaction.

Administration of bacterial polysaccharides (Pyrexal®) failed to elicit the expected rise in serum properdin in cancer patients.

Human properdin administered intravenously in doses of 500 units per Kg. or more to patients with properdin deficiency was detected only briefly (up to two hours) in serum. When similar doses were given daily for five days this disappearance from the blood stream became less rapid, and low levels of properdin remained detectable in serum for about a week thereafter. The data suggest that administered properdin was rapidly bound to as-yet-unidentified tissue sites rather than excreted or metabolized.

The present studies do not permit any evaluation of possible therapeutic effects.

The Role of pH Gradients in the Distribution of Ammonia Between Blood and Cerebrospinal Fluid (CSF), Brain, and Muscle. JAMES STABENAU, KENNETH S WARREN, and DAVID P. RALL, Bethesda, Md. (Introduced by Jacob Robbins).

A pH gradient normally exists between blood and cerebrospinal fluid, with blood about 0.1 units alkaline to CSF. Similar gradients presumably exist between blood and muscle or brain, and can influence the distribution of compounds which are partially ionized at body pH, since only the un-ionized form is freely diffusible. We have examined the distribution of one such compound, ammonia, between blood and CSF, muscle, or brain in anesthetized dogs infused with ammonium bicarbonate. The control CSF/blood ammonia ratio was 0.6; brain/blood ammonia was 0.8. After NaOH infusion blood pH rose to 7.7 while CSF pH remained essentially constant. With this larger gradient, CSF/blood ammonia rose to 1.3, and brain/blood ammonia became 2.0. HCl infusion lowered blood pH to 7.1; CSF pH again remained unaltered. With a reversed pH gradient CSF/blood ammonia fell to 0.4, and brain/blood ammonia became 0.9. Respiratory alkalosis and acidosis gave similar data, and muscle ratios were even more striking than brain ratios. If only un-ionized ammonia diffuses freely between blood and CSF, the ratio of total ammonia between these compartments is the in-

verse ratio of the percent un-ionized ammonia (which is pH dependent) in each compartment. Assuming that pH determines the CSF/blood ratios, "apparent free plasma ammonia" was calculated and used to show that the blood/tissue ratios obtained experimentally were to be expected from known estimates of tissue pH. These experiments indicate the variations observable between blood and tissue ammonia. Since ammonia toxicity presumably occurs within the tissues, recognition of the influence of pH gradients on ammonia distribution may allow estimation of brain ammonia concentration, and the role of ammonia in hepatic coma may be more clearly evaluated.

The Effect of Intravenously Administered Ceruloplasmin on Copper Absorption in a Patient with Wilson's Disease. IRMIN STERNLIEB, ANATOL G. MORELL, and I. HERBERT SCHEINBERG,* New York, N. Y.

Two features of the disordered copper metabolism which characterizes Wilson's disease are marked deficiency, or absence, of the plasma copper-protein ceruloplasmin, and abnormal absorption of orally administered copper. Several investigators have postulated that ceruloplasmin deficiency is the primary inherited defect which results in abnormal and excessive copper absorption. This paper reports results of experiments designed to test this hypothesis.

When radioactive copper^{64} is orally administered to normal subjects the serum concentration of copper^{64} rises promptly, falls and rises a second time several hours later. The initial copper^{64} appearing in the serum is essentially all loosely bound to albumin, while the second elevation in copper^{64} concentration is due to ceruloplasmin copper^{64}. The latter may result from *1*) copper^{64} incorporation in newly synthesized ceruloplasmin, and/or *2*) exchange of copper^{64} with copper in pre-existing ceruloplasmin.

When copper^{64} is administered to patients with Wilson's disease the initial rise and fall in serum copper^{64} concentration appear, but there is no second elevation.

A patient with Wilson's disease was given 2.3 mg. of copper as cupric^{64} acetate in each of two experiments two weeks apart. Twenty-one hours prior to the second experiment 1.35 Gm. of purified ceruloplasmin was administered intravenously and this produced a normal serum ceruloplasmin concentration of 25 mg. per 100 ml. Copper^{64} concentrations in serum and in ceruloplasmin were measured at intervals. In both experiments serum copper^{64} rose initially, and fell, but there was essentially no secondary rise and no copper^{64} in ceruloplasmin. In both experiments 42 per cent of the administered copper^{64} was recovered in stools and urine. These results suggest that: *1*) Exogenous ceruloplasmin does not alter the manner or amount of copper absorption in patients with Wilson's disease, and *2*) these patients seem unable to effect exchange of exogenous ceruloplasmin copper with orally administered copper^{64}.

The Existence of a Large, Slowly-Exchangeable Pool of Body Sodium. DAVID H. P. STREETEN, ABRAHAM RAPOPORT, and WILLIAM S. WILSON, Ann Arbor, Mich. (Introduced by Jerome W. Conn).

Injected radiosodium exchanges with a pool comprising 70 per cent of total body sodium in 24 hours. Observed expansion of this pool (Na_e) over the ensuing six days has been assumed to result from unmeasured extrarenal sodium losses. Sodium unexchanged at 24 hours has been considered relatively inert and unexchangeable.

In seven normal, young adult males, Na_e has been estimated from daily serum and urinary counts for one to four weeks after administration of 3 μc. $Na^{22}Cl$. Sodium intake was 6.9 mEq. per day in two subjects, to minimize extrarenal losses. Two others received constant, normal sodium intake, and were not allowed to wash for two periods totaling two weeks. Determinations of stable sodium and of radioactivity were performed on ashed stools and on concentrates of bath fluid used for the final washing of the body, clothing, plastic stockings and bed clothes. In the three remaining subjects similar studies of losses of sodium from feces and skin were restricted to four day periods.

Na_e increased exponentially throughout the study in all subjects, mean increments varying from 17.0 to 66.7 mEq. per day. These changes did not result from extrarenal sodium losses which were below 5 mEq. per day in six subjects and 6.8 mEq. per day in the seventh. Mean Na_e increased from 42.6 mEq. per Kg. at 24 hours to 50.8 mEq. per Kg. after two weeks. In two subjects, final values of Na_e approached reported estimates of the total amount of body sodium. Since the expansion of Na_e had not ceased at the end of the observation periods, it is concluded that most, if not all, body sodium is eventually exchangeable in young adult males. The slowly exchangeable component of the sodium pool is probably in bone. In studies of sodium metabolism the significance of a slowly versus a rapidly expanding Na_e is obvious.

Relation of Size of Ventricular Septal Defects to Circulatory Dynamics. H. J. C. SWAN, ROGER M. SAVARD, and JOHN W. KIRKLIN, Rochester, Minn. (Introduced by Earl H. Wood).

Ventricular septal defects were measured at operation in 43 and at necropsy in 2 patients in whom circulatory dynamics had been determined previously at cardiac catheterization. A critical area for such defects should exist above which pressures in the right and left ventricles would equalize, while less area would offer sufficient impedance to blood flow between the ventricles to prevent equalization of pressures.

Systolic pressures were significantly less in the right ventricle than in the left in 11 of 13 patients with defects of less than 0.9 sq. cm. per $M.^2$ of body surface and an inverse correlation was demonstrated between size of the defect and the left-to-right systolic pressure gradient. Systolic pressures were similar in the left and right ventricles of 21 of 24 patients with defects that exceeded 1.2 sq. cm. per $M.^2$, and this relationship did not vary as defect size increased. Calculated resistance to flow across

the defect (pressure/flow index) was related in the theoretically expected manner to defect area. The ratio of pulmonary to systemic vascular resistance (*i.e.*, the relative resistance to blood flow through the pulmonary vessels independent of body size) showed a poor positive correlation in patients with defects of 1.0 sq. cm. per $M.^2$ or less, and no correlation was evident in patients with larger defects. In this latter group there was no correlation between defect area and magnitude of left-to-right shunt.

It is concluded that in the absence of high pulmonary vascular resistance, ventricular septal defects of less than 1.0 sq. cm. per $M.^2$ offer sufficient impedance to flow across the defect to prevent equalization of right and left ventricular systolic pressures. Defects of larger size virtually equalize ventricular pressures, and other factors, chiefly pulmonary vascular resistance, determine pulmonary blood flow and intracardiac shunts.

Homografts of Bone Marrow in Dogs After Lethal Total-Body Radiation. E. DONNALL THOMAS, HARRY L. LOCHTE, JR., ALFRED JARETZKI, III, CHARLES A. ASHLEY, and OTTO D. SAHLER, Cooperstown, N. Y. (Introduced by Joseph W. Ferrebee).

Life-saving homografts of bone marrow after lethal total-body radiation usually succeed in rodents but fail in dogs. Presumably a major problem in the dog is an active immune mechanism relatively resistant to radiation. To overcome this resistance the following procedures have been employed: *1*) Radiation dose has been increased beyond the LD_{100} range. *2*) The dose has been divided and given over a three day period. *3*) Recipients have been splenectomized and given adrenocorticotrophic hormone (ACTH) before radiation. *4*) Both donors and recipients have been of the same breed. *5*) The dose of bone marrow has been kept above 5 billion cells.

By a combination of these procedures and with the use of antibiotics and good nursing care, successful marrow homografts have been produced in six of seven dogs given 800 to 1,200 r. Successful grafts are attended by a return to the peripheral blood of all three formed elements and by an appearance of female leucocytes in male dogs that received marrow from female donors.

Inhibition of immune mechanisms to the degree required for a successful homograft implies loss of other immune responses. Greatly increased liability to infection is observed in irradiated dogs even after the formed elements of the peripheral blood have returned to normal. For this reason, transplantation of splenic tissue from adult or fetal donors is being studied as a means for reestablishing immunologic responsiveness and resistance to infection.

The bearing of these studies upon work with irradiated patients and upon homografts of other organs will be discussed.

The Relaxation Pressure-Volume Diagram in Pulmonary Disease. ER YI TING and HAROLD A. LYONS, Brooklyn, N. Y. (Introduced by Perrin H. Long).

The relaxation pressure diagram for normal human subjects has been constructed by Rahn and his associates as a method for the study of the mechanics of respiration. No previous reports of its use in the study of pulmonary disease have been made.

This study concerns itself with the determination of the relaxation pressure-volume diagram (P_R) in patients who have three distinct forms of respiratory impairment, *i.e.*, obstructive disease, restrictive disease, and combined disease of the lung. The results in each type of pulmonary disease are compared to the results found in the normal subjects.

After the total lung capacity and its subdivisions were measured, the intrapulmonary pressure (P_R, the total pressure) and the intraesophageal pressure (P_L) were measured. Relaxation pressure-volume curves were constructed and a similar curve for lung tension was plotted, and by subtraction of lung pressure P_L from P_R, the thoracic wall pressure (P_C) was obtained. The data so obtained gave reproducible curves.

In the restrictive disease group, the pressure-volume diagram was markedly different from that of the normal subjects. The curves are flattened and shift downward and to the right. In patients with obstructive disease, for example, emphysema, there is also a marked alteration in the P_R curve. It assumes a concave shape with a shift upward and to the left. With combined obstructive and restrictive disease of the lung, the P_R curve is raised on a higher level than normal, but in an area lying between the obstructive and restrictive type of curves. It can be shown that these findings may be due to one of two factors or a combination of: *1*) alteration of the lung pressure (P_L), when the disease is parenchymal, or *2*) alteration of the chest wall curve (P_C), when the disease is of the chest wall as in trauma or kyphoscoliosis.

The study also demonstrated that the reduced compliance of the lung is due to either parenchymal or thoracic change.

The significance of these results will be discussed. An analysis of the contributing factors involved in the alterations of the mechanics of respiration has not been given the attention it deserves. Some of the data suggest that some current thoughts of the stress-strain properties of the lung may have to be modified.

Urinary Excretion of Delta-Aminolevulinic Acid in Various Hematological Disorders. GARSON H. TISHKOFF, WILLIAM MCFARLAND, and LOUIS J. FAENZA, Boston, Mass. (Introduced by William Dameshek).

The role of δ-aminolevulinic acid (ALA) as a precursor in the biosynthesis of heme has been recognized. This metabolite has also been demonstrated as a constituent of normal urine. On the assumption that the excretion of ALA is primarily a reflection of erythroid biosynthetic function, an attempt has been made to correlate urinary levels of ALA with bone marrow erythroid activity in various blood disorders.

Various hematological disorders affecting erythroid tissue were studied, including erythroid hyperplasia, ery-

throid hypoplasia, maturation arrest and malignant proliferation. The results are expressed as micromols of ALA $\times 10^{-6}$ per ml. of urine. The mean normal urinary level in 16 subjects was 30.5 ± 2.9 as determined on random urine specimens. Elevated levels of 150 to 350 were found in various porphyrinurias. These included one patient with acute intermittent porphyrinuria and three patients with lead intoxication. Patients with marked erythroid hyperplasia as a result of hemolytic disease, such as hereditary spherocytosis, Cooley's anemia and acquired hemolytic anemia, evidenced values in normal or near normal range. A case of untreated pernicious anemia with maturation arrest also showed a normal urinary level of ALA.

Low values of ALA were seen in 14 patients with hypoplastic anemia. In these, urinary levels ranged from 1.7 to 12.0. Four patients with myelofibrosis and myeloid metaplasia showed decreased values ranging from 3.3 to 6.6. Low values of ALA were also observed in patients with malignant proliferation, either as a result of acute leukemia, erythroleukemia or metastatic carcinoma.

It is suggested that determinations of urinary δ-aminolevulinic acid may be of value in assessing the degree of bone marrow injury in such conditions as hypoplastic anemia, including those due to large doses of ionizing radiation, and myelofibrosis.

The Effect of pH on Norepinephrine-Induced Contractions of Arterial Smooth Muscle. Louis Tobian,* Stephen Martin, and William Eilers, Minneapolis, Minn.

Spirally cut strips of rat aorta were mounted under 0.5 Gm. tension in Krebe-Henseleit medium with 25 mEq. of bicarbonate per liter. The bath was alternately aerated with gas containing either 4.5 or 11 per cent CO_2, giving pH's of 7.5 and 7.2, respectively. These alternating changes of pH did not by themselves change the tension of the strips. Then norepinephrine (10^{-6}) was added to the bath at pH 7.5 and the increased tension was measured isometrically. Then the norepinephrine was washed out, allowing the tension to return to the 0.5 Gm. baseline; the pH was changed to 7.2 and norepinephrine (10^{-6}) was again added. These alternations of pH were continued consecutively for five successive contractions in each of six strips. At pH 7.5 the strips developed an average additional tension of 0.30 Gm. with norepinephrine, while at pH 7.2 the average additional tension was only 0.17 Gm. The acid medium caused an inhibition of contraction in every strip, averaging 43 per cent inhibition. In a similarly arranged study with six other strips, the pH was alternately changed from 7.5 to 7.2 by alternating media with either 28 mEq. or 14 mEq. of bicarbonate per liter, respectively, while keeping the CO_2 constant. Again the average increase in tension with norepinephrine at pH 7.5 was 37 per cent greater than that at pH 7.2. In three other strips the pH was alternated between 7.35 and 7.15 by changing the bicarbonate concentration while keeping the CO_2 constant. The increase in tension with norepinephrine was 38 per cent greater at 7.35 than at 7.15. Summarizing, in every strip of every study the contraction was invariably diminished in the more acid medium ($p = 0.00001$). This would suggest that acidotic patients can be rendered more sensitive to norepinephrine by correcting their acidosis.

Effects of "Dry" Heat Exposure on Splanchnic Flow and Resistance in Man. Elmerice Traks and Salvatore M. Sancetta, Cleveland, Ohio. (Introduced by George J. Gabuzda).

Previous observations have shown that an elevation in the ambient temperature produces musculo-cutaneous vasodilatation. Our data have demonstrated decreases in the brachial and pulmonary artery pressures, total systemic and pulmonary resistances, with no change in cardiac output in man during a two hour exposure to ambient temperature of 37° C. and relative humidity of 40 per cent. It has been generally assumed that this vasodilatation is accompanied by visceral vasoconstriction. The following experiments were designed to test this hypothesis.

Splanchnic blood flows (BSP extraction) were determined in a group of six subjects during a two hour exposure to 23° C. and 40 per cent humidity. These served as controls for the method. In 10 subjects, following basal determinations at the above environment, flows were determined at the end of one and two hours' exposure to ambient temperature of 37° C. and humidity of 40 per cent. Both groups included patients with normal and enlarged left ventricles.

The group of subjects observed at the comfortable environment had average flows of 746, 772, and 721 ml. per $M.^2$ per minute, and splanchnic resistances of 7.6, 7.7, and 7.9 resistance units for the initial, one hour and two hour determinations, respectively. Those exposed to heat had average flows of 731, 778, and 741 ml. per $M.^2$ per minute, and resistances of 9.0, 7.5, and 5.9 units for the control, one hour and two hour periods. Only the changes in resistance in the latter patients were statistically significant ($p = < 0.001$). Three of the ten patients exposed to heat were in left ventricular failure; their data did not differ from the other seven not in failure.

The data indicate that vasodilatation rather than vasoconstriction occurs in the splanchnic bed when ambient temperature is acutely elevated and humidity is maintained at a relatively low level.

Production of Free and Conjugated Corticosteroids by Perfused Human Placentas. Philip Troen, Boston, Mass. (Introduced by A. Stone Freedberg).

The contribution of the human placenta to synthesis or metabolism of corticosteroids has not been clear. Indirect evidence has been adduced for placental production of corticosteroids as a cause of the elevated plasma corticosteroid levels observed during pregnancy. Other workers, however, have attributed this change to decreased corticosteroid metabolism.

Perfusion of the human placenta by a technique developed in this laboratory seemed a promising direct ap-

proach to this problem. Validity of the methodology has been supported by our recent demonstration of continuing placental metabolism in the form of citrate utilization during perfusion for 12 hours.

Ten placentas from normal pregnancies were obtained immediately after delivery and perfused with a modified Tyrode's solution containing citric acid. Seven placentas were perfused 12 hours, two 8 hours, and one 6 hours. The perfusate was analyzed for free and conjugated corticosteroids using dichloromethane extraction, β-glucuronidase hydrolysis, and the Porter-Silber (P-S) reaction.

Mean cumulative production (in cortisol equivalents) of total P-S material per placenta during perfusion for 12 hours was 8.2 mg. (range, 0.75 to 14.9 mg.). Mean hourly production per placenta increased linearly from 0.13 mg. during the first hour to 1.5 mg. during the twelfth hour. Mean cumulative ratio of free to conjugated material was 2 (range, 0.15 to 4.5). Extraction of placental homogenates yielded less than one-twentieth the amount obtained from perfusates, thus ruling out release of preformed P-S material.

The nature of the P-S material was studied. Ethyl acetate extracts of perfusates were analyzed by countercurrent distribution (3 solvent systems, 100 tube apparatus) and by paper chromatography (Bush B-5 system) combined with ultraviolet absorption, P-S reaction, and blue tetrazolium staining. Preliminary results are consistent with the presence in perfusates of cortisone, cortisol, tetrahydrocortisone, tetrahydrocortisol, compound S and as yet unidentified polar material.

These results indicate the human placenta synthesizes and conjugates corticosteroids.

Platelet Thromboplastic Factor: Its Chemical Nature, In Vitro Activity, and the Identification of Similar Thromboplastic Substances in Red Blood Cells. STANLEY B. TROUP and CLAUDE F. REED, Rochester, N. Y. (Introduced by S. N. Swisher).

A substance supplied by normal platelets is required for the formation of thromboplastin during the clotting process. This substance is known to be or to contain phospholipid, and it has been suggested that it is a single phospholipid, phosphatidylethanolamine. Quick has also demonstrated the presence of a factor in normal red blood cells that can contribute to thromboplastin formation.

Methods recently developed allow the extraction and separation of the unaltered lipids from platelets and red cells, and the identification and measurement of the components. The component lipids from platelets and red cells have been isolated, highly purified, and their thromboplastin generating activity measured.

The following observations have been made: *1*) The composition of the lipid fraction of platelets and red cells is similar. *2*) The red cell lipid fraction contributes to thromboplastin generation and probably is the red cell factor described by Quick. Red cell phospholipids are fully as active as those found in platelets. *3*) Of the various phospholipids, only phosphatidylserine gives maximal thromboplastic effect at a concentration of 2 micrograms per ml. of platelet-free plasma. Phosphatidylethanolamine can also give a maximal effect, but requires a much larger amount. *4*) One microgram of the total lipid extract per ml. of platelet-free plasma augments thromboplastin generation although this extract contains only 7 per cent phosphatidylserine.

The unique role of phosphatidylserine and phosphatidylethanolamine as cellular components of the thromboplastin generation system is suggested by their virtual absence from normal plasma. The hemorrhagic tendency or thromboses frequently seen in disorders involving intravascular hemolysis may be related to the release of the lipid substances from the lysed red cells.

Hypercalciuria Associated with Reduction in Corticoid Therapy After Prolonged Administration of Prednisone and After Bilateral Adrenalectomy for Cushing's Syndrome. STEWART G. TUTTLE and WM. G. FIGUEROA, Los Angeles, Calif. (Introduced by John S. Lawrence).

This study documents exchanges of calcium, phosphorus and nitrogen during the recovery phase of two men with Cushing's Syndrome. Disease in the first resulted from three years' therapy of pemphigus vulgaris with cortisone and prednisone; the second was a case of spontaneously occurring bilateral adrenal cortical hyperplasia. Balance data have been obtained: 670 days in the first patient, and 225 days pre- and postadrenalectomy in the second. While on a constant regimen slow reduction of a dose of prednisone in Case 1 from 25 to 5 mg. per day resulted in a fall in urinary calcium and storage of nitrogen and phosphorus. However, at a dosage of 3 mg. per day, although there was no recurrence of skin lesions, symptoms of corticoid withdrawal were noted and were accompanied by hypercalciuria, increased fecal calcium, negative calcium balance and a small rise in serum inorganic phosphorus. These were controlled by increase in the dose of prednisone to 5 to 7 mg. per day. Findings similar to the above were observed during cortisone replacement therapy in the postoperative studies made on the second patient. When receiving 75 to 100 mg. cortisone per day calcium, phosphorus and nitrogen were retained and urinary calcium was normal. Reduction of the dose of cortisone to 37.5 mg. per day produced mild symptoms of adrenal insufficiency, calciuria of 400 to 500 mg. per day and an increase in fecal calcium. The balance of nitrogen remained strongly positive. Hypercalciuria disappeared when 75 mg. per day dose of cortisone was restored. The complete dichotomy between tissue synthesis (positive nitrogen balance) and loss of calcium in this latter instance seems to cast doubt on the hypothesis that a protein anabolic effect necessarily protects the skeleton against decalcification. Apparently a deficiency as well as an excess of cortical hormones may affect the calcium balance adversely.

The Use of a Patient-Cycled Respirator to Evaluate Mechanical and Central Factors in the Hypercapnia of Emphysema. JOHN M. TYLER and BIRGER GRAPE, Boston, Mass. (Introduced by Thomas C. Chalmers).

The relative importance of mechanical versus central factors in the ventilatory drive of nine emphysematous hypercapnic patients has been studied, using a patient-cycled respirator. A signal proportional to air-flow (obtained by electrical differentiation of a signal from a pneumograph around the patient's chest) is led to a servo-amplifier which controls a valve between a blower and the body respirator, producing an applied pressure proportional to flow. Since this affords continual assistance in overcoming flow resistance and thereby reduces the work of breathing, it would be anticipitated that the ventilatory response to hypercapnia, both resting and increased by inspired CO_2, would be augmented to the extent that mechanical factors limit this response.

The ventilatory response to inspired CO_2 was tested during unassisted and assisted breathing. Arterial pCO_2 (Pa_{CO_2}) was estimated by the rebreathing method of Collier (mean difference in 22 simultaneous comparisons with arterial pCO_2, $+0.4 \pm 2.5$ mm. Hg [1 SD]). Three patients showed a marked augmentation in response to CO_2 during assisted breathing. In these patients, assistance during room air breathing caused hyperventilation. One patient had a severely depressed response to CO_2 which was not changed by assistance. While breathing room air, assistance caused his minute ventilation (V_E), oxygen consumption (V_{O_2}), and carbon dioxide production, (V_{CO_2}), to decline without altering Pa_{CO_2}. It is concluded that in this patient both resting ventilation and the response to inspired CO_2 were governed by a depressed respiratory center, while in others mechanical factors may be a determining influence.

In three patients assistance caused a significant, sustained fall in V_E, V_{O_2}, and V_{CO_2}. Despite the fact that estimated V_A was unchanged or decreased, Pa_{CO_2} declined 7 mm. Hg in two of these. This is consistent with the suggestion of Riley that the metabolic cost of breathing may play an important role in the hypercapnia of emphysema.

Specific Desensitization of the Delayed Hypersensitive State. JONATHAN W. UHR, New York, N. Y. (Introduced by H. S. Lawrence).

The delayed hypersensitive state can be induced to specific protein antigens in guinea pigs or in man by intradermal injection of minute amounts of antigen in the form of an immune precipitate. The degree of sensitization is enhanced if killed tubercle bacilli are incorporated into the suspension containing the specific precipitate. Active antibody production is suppressed under these conditions so that detectable antibody does not appear for two or more weeks after delayed skin reactivity has appeared.

In the present study guinea pigs were sensitized to two immunologically distinct proteins. One to two weeks later after maximal sensitization had been attained the animals were injected with a relatively large amount of one of the antigens. Afterwards, the animals were skin tested with 3 to 5 μg. of both antigens. Skin reactivity to the antigen which had been used for desensitization was specifically and completely abolished, but the same animals retained their skin reactivity to the second antigen to which they were sensitive. Although 2 mg. of specific antigen was required for complete desensitization, as little as 20 μg. sufficed to decrease the size of specific skin reactions in sensitized animals. The duration of the nonreactive state increased as larger amounts of desensitizing antigen were used. The return of delayed skin reactivity was usually associated with the appearance of circulating antibody. Desensitization could be accomplished by the specific antigen-antibody complex as well as by "free" antigen. The appearance of delayed skin reactions could be prevented in fully sensitized animals even when the desensitizing dose was given one or more hours *after* skin testing.

The ease of desensitization in these experimentally sensitized animals contrasts with prior studies reported in the literature in which specific desensitization of animals or man infected with tubercle bacilli was seldom successful.

Respiratory Adaptations in Induced Hyperthyroidism. HEINZ VALTIN and S. MARSH TENNEY,* Hanover, N. H.

In hyperthyroidism there is not only an increased tissue metabolic demand for oxygen but also a need for increased elimination of carbon dioxide. The respiratory apparatus could adapt by raising alveolar ventilation, or carbon dioxide tension, or both; the tissue need could be met by increasing blood flow, or lowering venous oxygen tension, or both.

In order to assess the individual factors which determine either full adaptation or compromise, hyperthyroidism was induced in normal human subjects and albino rats by the administration of triiodothyronine.

In man, a mean increase in basal oxygen consumption of 35 per cent was attended by an equivalent rise in alveolar ventilation with resultant maintenance of normal alveolar gas tensions and respiratory exchange ratio. Ventilatory stimulus-response curves derived by varying concentrations of carbon dioxide in inspired air indicated no significant increase in "sensitivity" of the respiratory center during hyperthyroidism.

"Tissue-venous" gas tensions in rats were approximated by the subcutaneous air pocket technique. For a thyroid-induced mean increase in oxygen consumption of 50 per cent the carbon dioxide tension fell 2.5 mm. Hg and the oxygen tension rose 8 mm. Hg. Tissue gas tensions, when related to rat whole blood respiratory exchange ratio isopleths, reveal deviations from the mean line suggesting increase in tissue blood flow : oxygen consumption ratio.

These data may be interpreted as showing that, for the degree of induced hyperthyroidism studied, respiratory adaptation to the increased oxygen consumption and carbon dioxide production is complete. The tissue-venous gas tensions indicate that regional blood flow has overcompensated tissue oxygen need.

Significance of Virulence and Viability as Related to Quantitative Immunogenic Properties of Several Antituberculosis Vaccines. H. M. VANDIVIERE, Chapel Hill, N. C. (Introduced by H. S. Willis).

Standardization for potency and viability in suspensions of Mycobacteria has long been an enigma of import, both requiring prolonged periods and measurable in retrospect only. Cord-formation titration, omental index (potency) and formazan curve interpolation (viability) are described, with corroboration, as standardization methods. Several vaccines have been so standardized and their immunogenic capacities quantitatively investigated.

"Degree of protection" has challenge of "protected subjects" as the final critique. Thus, 3,000 guinea pigs were randomly vaccinated with homogenous suspensions of one of the several vaccines which were standardized before use. Experimental groups were characterized so that statistical significance would evolve from either constant viability with potency, the protean factor or conversely. Tuberculin testing of experimental and control allocates with volume of allergic response $[v = 1/6\ h\pi(h^2 + 3r^2)]$ and duration of allergy calculations were periodic. Ultimately quantitative challenge was accomplished.

Effect of time on immunity was studied by postvaccination challenge at disparate periods from 6 to 30 months. Short-term vaccination reveals negligible differences in protection conferred by the various vaccines, whereas groups challenged at 12 to 30 months present significant differences between strains. The weakest BCG afforded little more protection than no vaccine. Degree of protection by R_1Rv is significantly higher than that conferred by the best BCG. Increasing potency and/or viability evokes increasing protection, but increased viability will not completely compensate for potency nor is protection as long duration. Evaluation of disease is based on Feldman's index and comprehensive pathological evaluations from coded gross and microscopic preparations.

Prevaccination cord-formation titer and omental index with postvaccination volume of allergic response, duration of allergy and degree of protection stand in comport for each strain. The weakest vaccine gives the least response, the most potent evokes greatest response and the intermediates between, each having its own specific level.

Beginning human studies (2,500) are presented on high-incidence groups.

The Rheumatoid Factor in Rheumatoid Arthritis. JOHN H. VAUGHAN and JEAN HARRIS, Richmond, Va. (Introduced by John L. Patterson, Jr.).

The rheumatoid agglutinating factor is a protein migrating electrophoretically as a fast γ-globulin. It is capable of agglutinating antibody-coated cells and of precipitating with Cohn Fraction II. Studies in this laboratory have emphasized its sharp specificity, as evidenced particularly by its ability to react with cells sensitized only with certain human anti-Rh antibodies. The reaction with rabbit antibody or gamma globulin has been shown to follow the pattern of reaction of an antibody for its cross-reacting antigen.

With the demonstration (Franklin *et al.*) that the rheumatoid factor exists in plasma as a complex of a heavy γ-globulin (19S) with a smaller γ-globulin (7S), attempts were made at its separation by filtration. Using a membrane with a maximum pore diameter of 0.1 μ, the factor was found to be retained, while uncomplexed γ-globulin passed through. The isolated product has the electrophoretic mobility of a fast moving γ- or a β-globulin. It contains carbohydrate to about 5 per cent of the total protein. Agglutination of sensitized cells can be induced by 0.1 to 0.5 μg. N of the material.

After transfusion of a normal individual with 250 ml. of rheumatoid plasma, the factor could be detected in the recipient circulation for two weeks. On repeated weekly transfusions of 250 to 500 ml. for six weeks there was no evidence of the development of an accelerated rate of disappearance of the factor, indicating that the factor was not treated by the recipient as a foreign antigenic substance. There was no evidence of the development of arthritis in the recipient, even when an attempt was made to provoke such reaction by injecting sterile saline into the knee.

The data are regarded as strongly indicating that the rheumatoid factor is antibody primarily directed to a human gamma globulin, cross-reacting with rabbit γ-globulin, and developing because of autoimmunization secondary to the underlying disease.

A Biochemical Study of Certain Skeletal Muscle Constituents in Human Muscular Dystrophy. PAUL J. VIGNOS, JR., Cleveland, Ohio. (Introduced by Walter H. Pritchard).

Progressive muscular dystrophy is the most common primary myopathy and may be regarded as the prototype of primary degenerative muscle disease. The skeletal muscle of 17 ambulatory dystrophics with relatively mild disease was compared with 10 normal controls and a second control group of 8 patients with neurogenic muscle atrophy to determine whether biochemical alterations found are specific. Myosin and enzyme determinations were referred to a parenchymal tissue base.

Degeneration of muscle in progressive myopathy is associated with the appearance of connective tissue and fat cells. Diagnostic significance is attached to the finding of high muscle fat content by histologic examination in dystrophy. However, quantitative muscle fat determinations show neither increase nor significant difference from neural atrophy. A significant increase in collagen nitrogen was found in all diseases studied. This appears to be a more dependable indication of early muscle dysfunction than fat deposition. Water content in experimental muscle atrophy has shown varying results. In the clinical groups studied, juvenile dystrophy showed the only significant reduction in muscle water content.

Muscle glycolysis shows the greatest change in muscular dystrophy. A marked reduction to one-third of the normal values occurred. Two nonglycolytic enzymes in-

volved in phosphate energy transfer, creatine kinase and adenosine triphosphatase showed moderate statistically significant decreases. Succinic dehydrogenase was normal.

A defect in contractile protein of muscle has been suggested as an alternate etiologic concept in dystrophy. The content of the principal myofibrillar protein, myosin, showed an absolute decrease proportional to the reduction in nonglycolytic enzymes.

Previous work has shown that glycolysis is impaired in dystrophy. These data give further support to a serious glycolytic defect but suggest, also, that the biochemical alterations are much more complex and involve both myofibrillar and sarcoplasmic components. The biochemical changes lack specificity but may represent a stereotyped response to dissimilar metabolic errors.

Effect of Sulfate on Physical State and Renal Excretion of Divalent Cations. MACKENZIE WALSER and ANN BROWDER, Baltimore, Md. (Introduced by Gilbert H. Mudge).

Recent physicochemical studies have established that bi-bivalent salts are incompletely dissociated, *e.g.,* the dissociation constant at 40° C. and ionic strength 0.15 is 0.004 for $CaSO_4$ and 0.005 for $MgSO_4$. The concentration of free divalent ions in plasma and urine may therefore be influenced by the concentration of divalent ions of opposite charge. If tubular reabsorption operates upon free ions, the excretion rate of calcium and magnesium should be influenced by the filtrate concentration of sulfate and phosphate. The converse should also apply.

During sodium sulfate infusion in unanesthetized dogs, the ultrafiltrable fraction of plasma calcium and magnesium (determined at 37° C. and pCO_2 40 mm. Hg) increased from 40 to 65 per cent in controls to 70 to 95 per cent, without hypoproteinemia. Tetany occurred despite normal plasma calcium and magnesium when plasma sulfate exceeded 20 mM per L., but could be prevented by inducing hypercalcemia or hypermagnesemia.

The fraction of filtered calcium and magnesium excreted increased from less than 3 per cent and less than 6 per cent, respectively, in controls to 70 per cent with sulfate infusion alone. When plasma chloride was reduced further by peritoneal dialysis with sodium sulfate, 75 to 105 per cent of filtered calcium and magnesium were excreted at normal filtration rate, and creatinine U/P of 2 to 3. These results were nearly independent of variation of plasma calcium from 5 mg. per cent to 24 mg. per cent and variation in plasma magnesium from 2 mg. per cent to 9 mg. per cent.

These findings are pertinent to the reduced protein-binding of calcium in uremia, the therapeutic use of parenteral $MgSO_4$, and the treatment of hypercalcemia and abnormal accumulations of other divalent ions.

A New Bactericidal System in Normal Serum and Plasma. RALPH J. WEDGWOOD, Cleveland, Ohio. (Introduced by W. M. Wallace).

Serum of man and other mammals contains many bactericidal factors presumably related to natural resistance to infection. One, the properdin system, lyses various bacteria, inhibits some viruses and interacts with endotoxins. Another bactericidal system, similar to but distinct from the properdin system, has been found. This system, bactericidal against certain Gram-negative bacteria, has been found in sera and plasma of all mammals tested. Greater activity is found in plasma than serum. The system is heat labile in serum, more stable to heat in plasma. In addition to bactericidal activity, under certain conditions the system induces a transient, nongenetic change in colonial morphology of the test organism resembling transformation from rough to smooth.

Both effects are induced by "endpiece" of complement (supernate following dialysis, pH 5.6, ionic strength 0.02) and hydrazine or ion-exchanged plasma. The system thus appears distinct from the first or fourth components of complement, and from euglobulins, and does not require magnesium, calcium or properdin. Factors resembling the second and third components of complement may be needed.

This system has similarity to a system inactivating endotoxin (Landy) and also to a system cytotoxic for certain cell lines in tissue culture (Bolande). Thus, serum and plasma appear to contain at least two distinct systems which in addition to their bactericidal activity may interact with endotoxin. Since endotoxins and tolerance to endotoxins appear related to resistance and susceptibility to various traumata including Gram-negative infections and shock, the occurrence of serum systems interacting both with bacteria and endotoxins is of biologic interest.

Morphologic Changes in Dogs Following the Production of Shock with Endotoxin and Their Comparison to the Morphologic Changes Occurring During Shock Associated with Bacteremia in Patients. MAX H. WEIL, Minneapolis, Minn. (Introduced by Raymond E. Weston).

Pathomorphologic studies were made in 10 dogs after production of a fatal form of sudden circulatory collapse which followed a single intravenous injection of endotoxin derived from Gram-negative bacteria. Principal alterations involved the liver, gall bladder and intestinal tract. These included acute hepatic congestion, marked edema and hemorrhage of the gall bladder wall, and intestinal mucosal hemorrhages and ulcerations. Vascular lesions consisting of deposits of intraluminal and subendothelial hyaline fibrinoid material were observed in small blood vessels in the mucosa and submucosa of the intestine, in the efferent venules of the liver, and in the small branches of the pulmonary arteries.

For comparison, a review was made of the autopsy findings in 19 consecutive patients who died of bacteremia and shock in the University of Minnesota Hospitals. The principal lesions included bronchopneumonia and abscess formation, supperative pyelonephritis and abscesses, congestive splenomegaly and hepatomegaly and intestinal mucosal hemorrhages and ulcerations. Hyaline thrombi were observed in the lumina of coronary arteries, pulmo-

nary arteries, mucosal and submucosal blood vessels of the intestine and in the adrenal sinusoids.

The most noteworthy feature was the regularity with which lesions occurred in the gastrointestinal tract, in the patients as well as in the animals. In the dogs, previous studies had shown that the immediate reaction to endotoxin is brought about by obstruction of outflow of blood from the liver. Consequently, large quantities of blood are pooled in the liver and portal venous bed and shock ensues. The relationship between stasis of blood and the formation of hyaline thrombi or emboli has not been elucidated, as yet. It seems likely, however, that the deposition of intraluminal and subendothelial fibrinoid material would account for the localized ischemic lesions which occurred in both groups. These comparative studies indicate that parallel changes occur in human patients and in experimental animals.

Some Antileukemic Effects of a Sulfhydryl Inactivator Derived from the Active Principle of Garlic (Allium Sativum). AUSTIN S. WEISBERGER, Cleveland, Ohio.

Sulfhydryl (-SH) compounds have frequently been implicated in cell growth and division and may have a particularly important role in malignancies and leukemia. Agents which inactivate -SH groups often exert an inhibitory effect on malignant growth.

Allicin (allylthiosulfinic allyl ester) is a powerful bactericidal agent derived from garlic by the interaction of an enzyme and substrate present in garlic bulbs. This compound inactivates many -SH enzymes without affecting most other enzymes and combines readily with cysteine. Such reactivity with -SH compounds suggests that it may also have inhibitory effects on malignant growth. Accordingly, various alkyl thiosulfinic alkyl esters (methyl, ethyl, propyl and butyl analogs) were studied in animals. These compounds inhibit the growth of Sarcoma 180 in mice. They also produce a lymphopenia and decreased cellularity in lymph nodes when administered to mice for 10 days.

In vitro effects on human leukocytes include a progressive decrease in the influx of S^{35} L-cystine with increasing concentrations and depression of both aerobic and anaerobic glycolysis.

Some effects of synthetically prepared diethyl and dipropyl thiosulfinic esters have been observed in four patients with leukemia. One child in the terminal resistant phase of acute leukemia manifested a fall in leukocyte count and marked decrease in spleen size. The spleen had previously not responded to other therapy. One patient with advanced chronic myeloid leukemia exhibited a rapid fall in immature leukocytes and decrease in spleen size. These patients received 100 to 400 mg. of these compounds for eight days. One patient with monocytic leukemia and one with chronic lymphocytic leukemia exhibited no significant response in five days.

Duration and amount of therapy as well as effects of other analogs require further evaluation. However, these preliminary results indicate that such compounds (R-SO-S-R) capable of inactivating -SH groups merit further study in leukemia and related disorders.

Tissue Electrolytes in Hypertension Due to Excess Sodium Chloride Administration. JOHN M. WELLER, Ann Arbor, Mich. (Introduced by Sibley W. Hoobler).

The prolonged administration of excessive amounts of sodium chloride (as 2 per cent in their drinking water) to rats resulted in the inconstant occurrence of hypertension. Analysis of the tissues of a group of rats which developed more severe hypertension showed significant differences from that of a group which had virtually no hypertension. The hypertensive rats had marked hypernatremia and hyperchloremia which were not present in the normotensive ones. These changes were not simply due to dehydration as the extracellular phase, as calculated for skeletal muscle, was actually expanded. The skeletal muscle of hypertensive rats contained more sodium and less potassium than did that of the more normotensive animals. Heart muscle of the hypertensive group showed predominantly a potassium deficit. In the aorta of the hypertensive rats, the major finding was an elevation of the sodium content. In contrast to this the aortas of rats not developing hypertension showed some increase in both sodium and potassium content.

From these data it would appear that excess tissue sodium and expansion of the extracellular space need not, in themselves, lead to elevation of the blood pressure. When severe hypertension occurred, potassium deficiency was the distinguishing additional finding. These observations again stress the importance of the maintenance of a normal sodium : potassium ratio and the physiological significance of the antagonistic action of these ions.

Studies on the Nature and Incidence of Buerger's Disease. STANFORD WESSLER,* SI-CHUN MING, VICTOR GUREWICH, and DAVID G. FREIMAN, Boston, Mass.

Fifty years after its classic description, Buerger's disease (TAO) has come under fresh scrutiny. Although TAO could not be established on clinical grounds, the diagnosis was considered in 79 of 1,464 Beth Israel Hospital patients with arterial insufficiency. Among 33 of these 79 patients there were 4 necropsies, 39 amputations, and 19 vein biopsies. Controls included 66 injected limbs amputated for atherosclerotic gangrene, 102 vein biopsies, and 23 dogs with experimental venous thrombosis. Lesions were classified pathologically as acute, intermediate, or healed according to Buerger's criteria. No acute arterial lesions were found although marked vasculitis with thrombosis resembling TAO was seen in gangrenous areas in both the TAO and atherosclerotic groups. The necropsies and amputated limbs from the TAO group revealed that the arterial occlusion was caused by thrombosis usually secondary to embolism or atherosclerosis. Frequently associated with these lesions were vascularization of the arterial wall, venous thrombosis, and perivascular fibrosis involving contiguous nerves. These findings, generally considered characteristic of the inter-

mediate and healed stages of TAO, were also found among limbs amputated for atherosclerotic gangrene. Moreover, some of the histologic venous features were reproduced in the animals. Finally, the phlebitis often considered typical of Buerger's disease was found among vein biopsies from both the TAO and control groups. These data indicate that the specificity of TAO cannot be established histologically by demonstrating acute arteritis in areas of gangrene, acute phlebitis, or intermediate or healed arterial lesions as defined by Buerger. These latter arterial lesions are nonspecific and can be produced by simple intravascular thrombosis. Since Buerger's disease cannot be precisely defined clinically, proof of the diagnosis becomes exceedingly difficult, for it depends on the unequivocal demonstration of the acute arterial lesion in an area free of gangrene. No patient at the Beth Israel Hospital has satisfied this criterion.

Hepatic Storage and Biliary Transport of Bromsulfalein in Dog and Man. HENRY O. WHEELER, JAY I. MELTZER, ROBERT M. EPSTEIN, and STANLEY E. BRADLEY,* New York, N. Y.

Hepatic mechanisms for removal of plasma constituents were explored using bromsulfalein (BSP) as a test substance. In 18 anesthetized dogs, hepatic BSP removal rate (R) was calculated from values for total hepatic venous outflow (rotameter) and splanchnic BSP arteriovenous differences under various conditions of loading produced by appropriate BSP infusions. The data appeared to support the view that hepatic removal of BSP (and presumably other substances) involves two processes: *1*) biliary secretion at a rate limited by a transfer maximum (Tm) equal to the fixed rate observed at any constant plasma level above 3 mg. per cent; and *2*) hepatocellular storage (S) in an amount proportional to plasma concentration. The second affects total removal rate only when changes in plasma level permit net storage uptake or release and may be computed as the change in removal rate observed under these circumstances relative to the rate of change in plasma level (mg. per mg. per cent).

Since extrahepatic removal proved to be small, Tm and S could be measured without determination of blood flow, by solution of simultaneous equations derived by substitution of values obtained during at least two different constant infusions of BSP into the following general equation:

$R = Tm + S \cdot (\Delta P/\Delta T)$, where $(\Delta P/\Delta T)$ equals the rate of change in plasma concentration and R equals the corresponding removal rate (infusion rate minus $(\Delta P/\Delta T)$ times plasma volume).

In 11 anesthetized dogs Tm averaged $1.73 \pm$ S. D. 0.45 mg. per minute per 10 Kg. body weight, and S averaged 19.7 ± 6.8 mg. per mg. per cent per 10 Kg. indicating a higher concentration of BSP stored in hepatic parenchyma than in plasma. In 13 normal human subjects Tm averaged 8.41 ± 1.32 mg. per minute per 1.73 $M.^2$, and S averaged 60.0 ± 26.1 mg. per mg. per cent per 1.73 $M.^2$. Abnormal patterns observed in hepatic disease included virtually complete absence of biliary transport accompanied by normal storage, suggesting that separate mechanisms are involved in storage and transport.

Inhibition of Mycobacteriophage Propagation by Fresh Serum. ARTHUR C. WHITE and VERNON KNIGHT,* Nashville, Tenn.

Fresh human serum has been found to prevent the propagation of mycobacteriophage D-29 on human tuberculosis strain $H_{37}Rv$. This effect was observed in sera from tuberculin positive and negative donors, and from patients with active tuberculosis in concentrations of 10 per cent and greater. The effect was not removed from fresh serum by pretreatment with living tuberculosis cultures, saprophytic mycobacteria, or mycobacteriophage. The inhibitory effect was completely removed by treatment of serum with trypsin. Alpha and beta globulin fractions prepared from serum by ammonium sulfate precipitation showed considerable effect; albumin and gamma globulin showed little effect. The active component could be distinguished from properdin and complement. This reaction superficially resembles the inhibition of mycobacteriophage propagation by Tween 80 previously reported from this laboratory, but may be distinguished by the failure of serum to prevent propagation of mycobacteriophage on the saprophyte, ATCC 607. The effect of serum appears to be on the bacterium rather than on the phage since serum does not prevent phage propagation on a saprophytic mycobacterium. Inhibition of phage propagation by serum may explain the observed failure of large doses of mycobacteriophage to modify experimental tuberculous infection in mice. It is probable that the lack of increase in lytic particles in phage-bacterial systems in the presence of serum indicates failure of phage to infect the bacteria. If this is so, this and similar phage-bacterial systems could not exhibit transduction and related phenomena *in vivo* (heritable changes produced in bacteria by infection with phage, such as increase in penicillin resistance and increase in virulence); nor could bacteriophage treatment be expected to exert a suppressive effect on animal infection.

The Influence of Adrenocorticotrophic Hormone (ACTH) on the Release of Nonesterified Fatty Acids from Rat Adipose Tissue In Vitro. J. EARLE WHITE and FRANK L. ENGEL,* Durham, N. C.

Oxycel and peptide ACTH possess adipokinetic and ketogenic activities when assayed in rats and mice, and it has been shown that these effects are neither mediated by the adrenals nor due to contamination with other hormones. The present study supports the hypothesis that ACTH influences fat metabolism directly by demonstrating an accelerated release of nonesterified fatty acids (NEFA) from adipose tissue on incubation with ACTH *in vitro*.

Twenty to 30 mg. portions of intra-abdominal adipose tissue from fasted male rats were incubated in 1 ml. of heparinized rat plasma at 36° C. in air in a Dubnoff shaking incubator. Plasma NEFA were determined be-

fore and three hours after the addition of hormone or a distilled water control. The results expressed as μM NEFA released per 100 mg. adipose tissue per three hours were as follows: control, $+0.19 \pm 0.06$ (S. E.); oxycel ACTH, 1 μg., 1.17 ± 0.21, 5 μg. 2.37 ± 0.28. Samples of protein ACTH, Corticotropin A, American Cyanamid fractions of α_1-α_2 δ-1 and γ and a product of pepsin digestion of β-corticotropin were all active whereas a chymotrypsin fraction was not. Exposure to 0.1 N NaOH at 25° C. for 24 hours did not affect activity, but boiling in NaOH for 20 minutes abolished it as did oxidation with H_2O_2; however, in the latter case activity could be restored by incubation with cysteine. These inactivation-reactivation procedures influence adrenal ascorbic acid depleting potency and other extra-adrenal metabolic activities of ACTH *in vivo* in the same manner. Somatotropic hormone (1,000 μg.), thyroid stimulating hormone (100 μg.), β-melanocyte stimulating hormone (400 μg.), oxytocin (Pitocin®) (1 U) and vasopressin (Pitressin®) (2 U) were inactive, whereas adrenaline and noradrenaline (1 μg.) were active but could be shown not to be responsible for the ACTH effect.

These results strengthen the hypothesis that ACTH influences directly the intermediary metabolism of extra-adrenal tissues (Yale J. Biol. Med. 1957, **30**, 201.).

The Mechanism of the Protein-Sparing Effect of Glucose. JEAN D. WILSON and MARVIN D. SIPERSTEIN, Dallas, Texas. (Introduced by Gladys J. Fashena).

The mechanisms by which glucose administration during starvation spares protein are unknown. Previous studies in our laboratory demonstrated that glucose oxidation can control lipogenesis by generating rate-limiting cofactors, particularly during the operation of the hexosemonophosphate shunt. The present study was undertaken to investigate the relationship between protein synthesis and the cofactors generated during glycolysis—TPNH, DPNH, and ATP.

The influence of cofactors and substrates on protein synthesis from acetate-C^{14} was studied in cell-free homogenates of rat liver. Addition of TPN plus glucose-6-phosphate (G-6-P) to homogenates increased protein synthesis three- to sevenfold; addition of DPN plus G-6-P stimulated protein synthesis to a lesser extent. Addition of an ATP-generating system (creatine phosphate plus ATP) also accelerated protein synthesis markedly. However, when protein synthesis was maximally stimulated by the ATP-generating system alone, addition of TPN plus G-6-P causes further protein synthesis up to threefold; again, the effect of DPN plus G-6-P was usually less. Furthermore, to establish that pyridine nucleotides generated during glycolysis are, in fact, limiting in protein synthesis, valine-C^{14} incorporation was studied. Again, TPN and DPN were found to stimulate protein synthesis above levels attainable with maximal ATP generation alone.

It is noteworthy that pyridine nucleotides can regulate protein synthesis, since the only reaction in mammals resulting in net amino acid synthesis (α-ketoglutarate to glutamate) requires reduced pyridine nucleotide. Thus, the rate of amino acid synthesis is probably rate-limiting in protein synthesis. The conclusion may also be drawn that the generation during glycolysis of the cofactors—TPNH, DPNH, and ATP—may account for the protein-sparing effect of glucose. In addition, the finding that TPNH is more limiting than DPNH suggests that the glucose oxidized via the hexosemonophosphate shunt may be of particular importance in the sparing of protein by glycolysis.

Depression by Growth Hormone of the Phosphogluconate Oxidative Pathway in Adipose Tissue. ALBERT I. WINEGRAD, WALTER N. SHAW, and ALBERT E. RENOLD,* Boston, Mass., and Philadelphia, Pa.

Paired preparations of rat epididymal adipose tissue have been adapted for the study of hormonal effects *in vitro*. Studies concerned with production of CO_2 and synthesis of long-chain fatty acids from glucose-1-C^{14} and glucose-6-C^{14} by adipose tissue from normal, fed animals suggested approximately equal participation of glycolytic and nonglycolytic pathways of glucose utilization. The presence of the phosphogluconate oxidative (PGO) pathway in this tissue was further supported by the demonstration of glucose-6-phosphate and 6-phosphogluconate dehydrogenase activities. Insulin added *in vitro* consistently stimulated glucose oxidation to CO_2 and fatty acid synthesis from glucose by *both* pathways. The effect of insulin in stimulating fatty acid synthesis from acetate or pyruvate was dependent upon concomitant stimulation of glucose utilization.

Bovine growth hormone added *in vitro* (0.2 to 1 mg. per ml.) consistently stimulated glucose uptake and oxidation to CO_2, but fatty acid synthesis was not increased. Furthermore, in the presence of growth hormone the ratio of carbon 1 to carbon 6 metabolized to CO_2 tended towards unity, strongly suggesting depressed glucose utilization by the PGO pathway. Failure to observe increased fatty acid synthesis concomitant with increased glucose uptake and oxidation in the presence of growth hormone and the observed depression in PGO pathway activity support the view that glucose utilization by the PGO pathway is essential for synthesis of fatty acids in adipose tissue. The different patterns of glucose utilization produced by growth hormone and insulin *in vitro* make untenable the view that this effect of growth hormone is dependent upon the activation of insulin bound to tissues. The relevance of these observations to the physiological action of growth hormone is yet to be ascertained in view of the high concentrations of growth hormone required.

Mechanisms of Body Fluid Enzyme Alterations in the Absence of Tissue Necrosis. FELIX WRÓBLEWSKI, ALICE E. MOORE, CARLOS MANSO, and KANEMATSU SUGUIRA, New York, N. Y. (Introduced by Olof H. Pearson).

In the absence of cellular necrosis, intracellular enzymes may leave the intact cells and find access into the body fluids. Using the technique of tissue culture, it has been

shown that growing cells with intact cellular boundaries contribute lactic dehydrogenase activity to the bathing fluid medium; other intracellular enzymes simultaneously measured are not lost from the intracellular confines. Experimentally produced malignant tumors in rodents are associated with increased serum glutathione reductase and serum lactic dehydrogenase activity at a time when the malignant tumor shows decreasing enzyme activity of the former, increasing the activity of the latter enzyme and no tissue necrosis. Other tissue enzymes measured are simultaneously unaffected by neoplastic tissue growth. Inflammation of liver tissue results in the loss of intracellular transaminating enzymes in amounts independent of the hepatocellular enzyme content and not proportional to tissue necrosis. Plasma phosphatase enzymes from osseous and prostatic tissue sources are independent of tissue concentration and of cellular necrosis.

In vitro and *in vivo* studies indicate that major alterations of body fluid enzymes may occur in the absence of tissue necrosis, and that intracellular enzymes of tissues involved by neoplastic and inflammatory pathologic states may find access to plasma, serous effusion, and cerebrospinal fluid in amounts relatively independent of tissue enzyme concentrations and in the absence of cellular necrosis. The clinical parallelisms and implications are noteworthy.

On the Regulation of Purine Nucleotide Synthesis and Uric Acid Production in Gout. JAMES B. WYNGAARDEN and HAROLD R. SILBERMAN, Durham, N. C. (Introduced by R. Wayne Rundles).

Patients with primary gout show prompt overincorporation of tracer doses of glycine-1-C^{14} into urinary uric acid. All known pathways yielding uric acid involve purine nucleotide intermediates. Therefore, overproduction of uric acid in gout probably involves excessive synthesis and rapid cleavage of nucleotides. Factors controlling purine nucleotide synthesis have been investigated in pigeon liver homogenate supernatants ($105{,}000 \times G$). *De novo* synthesis of inosinic acid from glycine-1-C^{14}, formate, glutamine, aspartate, bicarbonate, ribose-5-phosphate (R5P) and ATP was inhibited by a number of purine compounds. When glycine-1-C^{14} and R5P were replaced by glycineamide ribotide-1-C^{14} (GAR), none of these compounds was inhibitory; therefore, the inhibitory compounds could be studied in terms of their influence on reactions prior to GAR formation. Three potential feedback control mechanisms were defined, by which purine end-products might regulate *de novo* purine synthesis. Possibly the most important is one by which preformed purine bases compete for phosphoribosylpyrophosphate (PRPP) in the course of conversion to the corresponding 5'-nucleoside monophosphate, thus preventing utilization of PRPP for *de novo* purine synthesis. This suggested, as one possibility in gout, that a mechanism resulting in accumulation or overproduction of PRPP might permit, ultimately, excessive *de novo* synthesis of purine nucleotides. This possibility is being explored in partially purified extracts of erythrocyte acetone powders. In limited studies to date, however, enzyme preparations from gouty erythrocytes appear to produce PRPP from ATP and R5P at normal rates, and to utilize PRPP in conversion of hypoxanthine and guanine to nucleotide stages at normal rates. Furthermore, they also cleave inosine at normal rates. These preliminary observations on gouty erythrocytes have not disclosed a defect which might either overproduce or underutilize PRPP, nor one which might cleave nucleosides excessively. Observations on liver might be of more critical importance in relation to a possible biochemical lesion in gout.

Circulatory Dynamics of Aortic Stenosis. PAUL N. YU, FRANK W. LOVEJOY, JR., BERNARD F. SCHREINER, HEINZ WALTHER, ROBERT H. LEAHY, and C. ALPHEUS STANFIELD, Rochester, N. Y. (Introduced by Nolan L. Kaltreider).

Combined right heart catheterization and direct left ventricular puncture were performed in 14 patients with "pure" aortic stenosis.

These patients are classified into three hemodynamic categories: Group I–Little or no alteration of left ventricular function, clinically asymptomatic; Group II–Moderate alteration of left ventricular function, clinically symptomatic; and Group III–Left ventricular failure and pulmonary hypertension, clinically incapacitated.

In patients of the first two groups, aortic valve varied between 0.5 and 1.1 $cm.^2$, with normal or nearly normal cardiac index, and left ventricular end-diastolic pressure 15 mm. Hg or less. Systolic upstroke time was normal or slightly delayed. Pulmonary artery pressure and "left heart resistance" were normal or slightly elevated.

Patients in Group III had aortic valve areas of 0.4 $cm.^2$ or less with subnormal cardiac index and left ventricular end-diastolic pressure 25 mm. Hg or more. The systolic upstroke time was considerably delayed. Pulmonary artery pressure and "left heart resistance" were markedly elevated.

Left ventricular stroke work (LV_{SW}) was evaluated in relation to left ventricular filling pressure. In patients with a compensated left ventricle, the total LV_{SW} was markedly increased, and the effective LV_{SW} against pressure normal or increased. In those with a decompensated left ventricle the total LV_{SW} was normal or decreased and the effective LV_{SW} markedly decreased. In the latter patients, higher left ventricular filling pressure was required to perform the same work in comparison to the compensated patients.

Following intravenous injection of acetyl strophanthidin, distinct improvement in left ventricular function was observed in a patient of Group III, but no significant change was noted in three patients of Group I and II either at rest or during exercise.

Hyperpolarization of Muscle Membrane by Insulin. KENNETH L. ZIERLER,* Baltimore, Md.

There are several reasons for suspecting that insulin acts by association with muscle membranes and there is increasing evidence that insulin accelerates movement of

certain substances into and even out of muscle. Collapse of the membrane barrier to diffusion explains some insulin effects but fails to explain its role in potassium accumulation in muscle. This latter effect could be explained if insulin hyperpolarized muscle membranes. To test this hypothesis resting membrane potential of excised rat peroneus longus muscle was measured. Insulin, but not cysteine-inactivated insulin, hyperpolarized the membrane by about 8 per cent. There are two possible explanations for this hyperpolarization; it might be primary in the sense of a direct effect upon the membrane, or insulin might make potassium move into muscle by some other mechanism and the consequent shift in ratio of intra- to extracellular potassium would be responsible for the hyperpolarization. The latter possibility is less likely because the observed changes in potassium concentration were inadequate to account for the degree of hyperpolarization. It is proposed that insulin acts by distorting the membrane, simultaneously increasing its permeability to certain substances and increasing its resting potential. Potassium then moves to adjust its concentration ratio in response to the new membrane potential.

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